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From the Industrial Toxicology Research Centre, Chattar Manzil Palace,
Lucknow India

Effect of Intratesticular Injection of Lindane and Endrin on the Testes of Rats

By

T. S. S. Dikshith and K. K. Datta

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Abstract: Little is known about the action of different insecticides on the testicular tissue of animals. The administration of lindane and endrin into the rat testis produced hypertrophic and atrophic changes in the testis and induced total arrest of spermatogenesis. Unlike cytotoxic or alkylating agents, the action of these two insecticidal compounds was non-specific to any group of spermatogenic cells. Intertubular and intratubular regions of the testis showed degenerative changes, necrosis and cellular proliferation. The seminiferous epithelium of the tubules was completely damaged. Formation of multinucleate giant cells of the foreign body type reaction was very common following lindane treatment. The number of spermatozoa and the percentage of deformed (abnormal) spermatozoa was very low after lindane administration as compared to that of endrin.

Key words: Lindane – endrin – testes – degenerative changes.

Various physical as well as chemical agents are known to cause testicular atrophy and damage of the seminiferous tubules, culminating in the partial or total arrest of spermatogenesis in animals. The mode of action of the physical factors differ from that of chemical agents (HARRISON & OETTLE 1950 OETTLE & HARRISON 1952 STEINBERGER & DIXON 1959 CHOWDHURY & STEINBERGER 1964 STEINBERGER & TJIOE 1969). Again different classes of chemicals such as cytotoxic agents (KINDARD 1952 NELSON & STEINBERGER 1952 STEINBERGER *et al.* 1956 NELSON & PATANELLI 1965), alkylating agents (STEINBERGER 1962) amides (MAZZANTI *et al.* 1964 & 1968 STEINBERGER & SUD 1970) and metals (PARIZEK 1960 KAR & DAS 1962 CHANDRA 1971) show different types of actions. GAINES & KIMBROUGH (1964) showed that repeated doses of metatepa (5 mg/kg/day), when fed orally to rats, induce male sterility and testicular atrophy after about 77 days. There is an urgent need to gather more information on the

action of different insecticides on the testicular tissues of animals. In this report an attempt has been made to study the action of lindane (hexicidum NFN) and endrin on the testicular tissue of rats.

Materials and Methods

40 adult male rats (MRC strain with a body weight of 200–250 g) and aged 180 to 190 days were used in the present studies. The animals were kept on stock diet which included gram, bran and green leafy vegetables. All the animals were grouped under three categories. The testes of each of the 16 animals of the first group were administered 0.25 ml of saline (0.7 %) by intratesticular injection and this served as the control group. 12 animals of the second and of the third group were similarly injected with 0.25 *mg lindane and 0.25 mg of endrin in saline* respectively (lindane (purity 98 %) was obtained from Plant Protection Ltd., Research Dept., Yalding through ICI (India) Pvt. Ltd., New Delhi and endrin (*re-crystallized) from Woodstock Agricultural Research Centre, Kent, U.K.). After treatment for 10 days* all the animals were killed. The individual testis was weighed and fixed in formal saline and the paraffin sections cut and stained with haematoxylin-eosin.

Results

The testes of all the 16 animals of the control group appeared to be normal. There was no sign of atrophy or hypertrophy of the testis in any of the animals. The architecture of the seminiferous tubules together with interstitial cells was also normal (fig. 1 & 1A). In contrast to the above picture, analysis of the testis ten days after the inoculation of lindane and endrin showed certain morphological and cellular changes. While lindane produced testicular hypertrophy and atrophy only hypertrophy was produced by endrin. Each animal showed only one kind of change. *The* changes in the size of the testis became evident after the 3rd to 4th day of treatment and did not return to normal even after 10 days. But for the testicular atrophy *or hypertrophy all the animals looked healthy* and there was no mortality. The quantitative difference between the testis weight of the control, lindane and endrin group of animals is given in table 1.

Lindane

Out of the 12 animals which received lindane, 7 showed testicular hypertrophy and 5 showed atrophy.

Hypertrophic testes. The testes were swollen to approximately double their normal size and their weight also confirms this finding. Histopathology of the testis included massive degenerative changes of the seminiferous epithelium intratubular and intertubular regions. The *seminiferous* tubules showed a slight increase in size, massive degenerative changes

Fig. 1. Testis of rat (control) showing normal pictures of the seminiferous tubules and interstitium. Haematoxylin and eosin. Magnification × 115.

Fig. 1A. Enlarged view of fig. 1. Haematoxylin and eosin. Magnification × 460.

Table I

Testis weight (g) of rats after intratesticular injection of lindane and endrin.

Group	Days after injection	No. of animals	Mean testis weight	
			Right	Left
Control	10	16	0.854	1.035
Lindane (hypertrophic)	10	7	2.081	1.768
Lindane (atrophic)	10	5	0.529	0.641
Endrin (less hypertrophic)	10	12	1.267	1.095



Fig. 2. Hypertrophic testis of rat, 10 days after the injection of lindane. Note massive degenerative changes of the seminiferous tubules and increased proliferation of the interstitial tissue. Haematoxylin and eosin. Magnification × 115

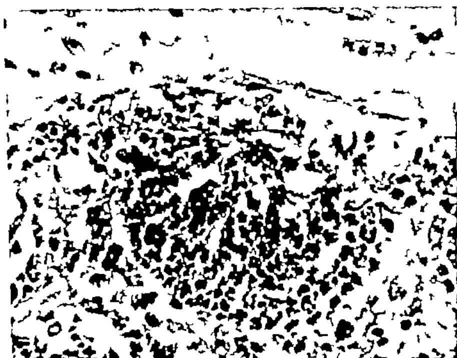
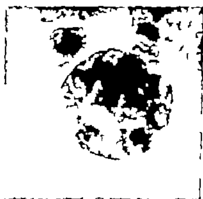


Fig. 2A. Enlarged view of one of the tubules to show the total damage to the architecture of the seminiferous tubules. Note the destruction of seminiferous epithelium. Haematoxylin and eosin. Magnification × 460.

were noticed in the spermatogenic cells. In some areas these cells were localised with oedematous fluid, polymorphonuclear cells and also a few deformed (abnormal) spermatozoa. Except for a few scattered resting spermatocytes the entire architecture of the tubules with different types of spermatogenic cells was totally damaged. Most of the tubules revealed the presence of numerous multinucleated foreign body types of giant cells. The interstitial tissue showed increased proliferation and was mixed with oedematous fluid. Polymorphonuclear cells were also present in the interstitial area (fig. 2 & 2A).

Atrophic testes: In the five animals which showed testicular atrophy the size of the testis was approximately reduced to ⅓ of its normal size. Histopathology of the testis revealed degenerative changes in the seminiferous epithelium and in different spermatogenic cells. The seminiferous tubules were shrunken and were always filled with oedematous fluid (fig. 3). The tubules consisted of a large number of multinucleated giant cells and acute inflammatory cells. The number of nuclei often varied from 10 to 30 (figs. 3A, 3B). The number of spermatozoa was greatly reduced. An in-

Fig. 3 Atrophied testis of rat, 10 days after the injection of lindane. Note the seminiferous tubules filled with oedematous fluid and multinucleated giant cells. Haematoxylin and eosin. Magnification × 160.





Figs. 3A, 3B. Enlarged view of the multinucleated giant cells carrying large number of nuclei. Haematoxylin and eosin. Magnification × 930.



Fig. 4 Atrophied testis (lindane) to show increase of interstitial space and proliferation of blood vessels. Haematoxylin and eosin. Magnification × 160.

crease of interstitial space and active proliferation of the blood vessels was observed (fig. 4)

Endrin.

Intratesticular inoculation of endrin did not induce atrophy of the testes. On gross observation the testes of all of the 12 animals showed near normal to slightly hypertrophied conditions (table 1) The stainability of the tissue was very poor All the spermatogenic cells of the tubules revealed massive necrosis which was more marked in the tubules near the capsule. Degenerated necrotic cells were mixed with oedematous fluid. Seminiferous epithelium and cellular architecture of the tubules was totally altered and the necrotic cells coalesced to form a mass. None of the tubules, however revealed the presence of any giant cells. A few of the

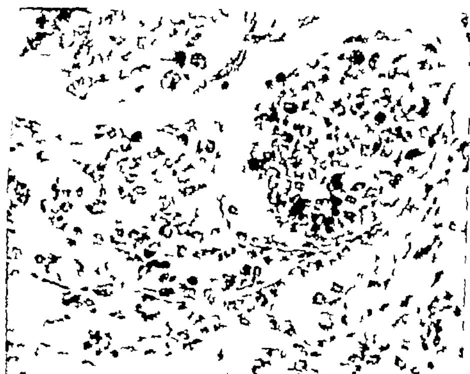


Fig. 5 Hypertrophied testis of rat, 10 days after the injection of endrin. Note the total destruction of the seminiferous epithelium, necrosis, increase of the interstitial tissue mixed with oedematous fluid and degenerated cells. Haematoxylin and eosin. Magnification × 460.

seminiferous tubules revealed the presence of deformed spermatozoa. The interstitial tissue showed proliferation often mixed with oedematous fluid and acute inflammatory cells. Thin walled small blood vessels were present in the interstitial area (fig. 5)

Discussion

Irrespective of the route of administration, certain chemicals induce degenerative changes in the testicular tissue of different laboratory animals. The route of administration of a chemical often varied e.g. oral (MAZZANTI *et al.* 1964 & 1968 STEINBERGER & SUD 1970), intratesticular (KAR & DAS 1962, Report Cancer Res. Centre, 1961–65 this report), intraperitoneal and subcutaneous (PARIZEK 1960 CHANDRA 1971) and intravenous (KETDARD 1952) It is very important at this level to know the route of administration of a chemical since this may decide whether the chemical used has a specific or non-specific action on different types of spermatogenic cells. This should be emphasized since in none of the intratesticular in-

jections (including this report) was there any specificity as to the mode of action of the chemical tested on any group of cells rather the entire tubular and intertubular areas suffered total and irreversible damage. While different chemicals exhibited selective action – there was no selectivity of action with regard to the two insecticides used here.

The investigations of PARIZEK (1960) showed that a single intraperitoneal or subcutaneous injection of $CdCl_2$ produced acute necrotic changes in the testes of rats, mice, guinea-pigs, rabbits and syrian hamsters. KAR & DAS (1962) also injected small quantities of $CdCl_2$ and $ZnCl_2$ directly into the testes of rats, rabbits, monkeys and goats. Both group of workers observed severe damage to the seminiferous epithelium which was irreversible. The observations showed that acute necrotic changes not only involved the seminiferous epithelium and the tubules but also the interstitial tissue. In both the areas the degenerative changes were total and permanent. These areas consisted of debris like cells.

The administration of lindane, unlike endrin produced multinucleated giant cells. Furthermore lindane induced both hypertrophy and atrophy of the testis, while endrin did not produce such a change. The underlying factor controlling this difference other than individual animal variation is at present not clear

Specific findings of autopsy either of animals or human subjects have not been reported from acute poisoning of insecticides (COBLE *et al.* 1967) The studies of GAINES & KIMBROUGH (1964) with metatepa showed that repeated oral administration produced sterility and testicular atrophy in rats.

The reaction of the testicular tissue as well as of different spermatogenic cells to a foreign chemical should be considered. The subtle changes observed in the present report after a single intratesticular injection of lindane or endrin have not been observed in any of the cases following oral or parenteral administration. Human fatalities due to insecticides have occurred both after massive exposure as well as from possible cumulative effects of repeated low doses administered either orally or parenterally Under such circumstances a correlation of the present findings with the observations in the human subject becomes very difficult. Further studies are in progress to investigate the action of different insecticides on testicular tissue when administered by different routes.

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From the Department of Toxicology Pharmacia AB, Uppsala, and the Institute of Zoology University of Uppsala, Uppsala, Sweden

Teratological Studies on Thalidomide in Rats

By

B. Göran Jonsson

(Received April 13, 1971 Accepted September 24, 1971)

Abstract Pregnant rats were given 100 mg thalidomide per kg orally during days 7–11 or 10–14 of gestation. The substance was prepared as a 1% suspension by homogenizing in 0.5% methylcellulose. The foetuses were delivered by Caesarean section and were examined for soft tissue and skeletal malformations. The results indicated a retardation of the development in the group treated during days 7 11. This was based on a lower mean weight per foetus and on a lower degree of ossification in the tail, vertebral column and sternum.

Key-words: Thalidomide – growth (body weight) – growth (bone development).

In 1961 when thalidomide was first suspected of inducing birth defects in human subjects (Lenz 1961) much work was done to reproduce these types of malformations in different animal species. Although early studies indicated a non-teratogenic effect of thalidomide in rats, subsequent investigators (Bignami *et al.* 1962 King & Kendrick 1962, Klein Obbink & Dalderup 1963 Mc Coll *et al.* 1963 Murphy 1963) reported embryotoxicity as well as teratogenicity

The purpose of these experiments was to study the teratogenic effect of thalidomide in rats when the drug was given during different stages of the gestation period.

Material and Methods

Virgin Sprague Dawley rats (Anticimex, Stockholm) weighing 270–290 g were used. During the test, the animals were fed on rat bread, fresh carrots and water *ad libitum*. The environmental conditions were: temperature 22 humidity 50% light 11 hours/day

The occurrence of copulation was indicated by the presence of spermatozoa in the vaginal smears. This day was considered as day 0 of the pregnancy

The mated females were divided into three groups and were treated as shown in table 1.

As thalidomide (kindly supplied by Astra Läkemedel AB, Södertälje, Sweden) is almost insoluble in water it was prepared as a 1% suspension by homogenizing in 0.5% me-

Table 1

Treatment schedule for mated females.

		Dose	Treatment days p.c. (post coitus)
I	Controls	10 ml vehicle/kg bodyweight	7–14
II	10–14	100 mg thalidomide/kg bodyweight	10–14
III	7–11	100 mg thalidomide/kg bodyweight	7–11

thylcellulose. The suspension was prepared daily and given orally in a volume of 1 ml per 100 g body weight.

The experiment was terminated on day 20 post coitus (p.c.) when the females were killed by chloroform inhalation and the foetuses were delivered by Caesarean section.

The number of implantations was determined by the method of SALEWSKI (1964). After delivery the foetuses were inspected for external anomalies and prepared for skeletal examination using the technique of CRARY (1962).

Student's t-test was used in the statistical treatment of the results. The tables show the mean value for each group with the standard error of the mean ($\bar{x} \pm$ S.E.M.). Statistically significant difference = $P < 0.05$.

Results

The results are presented in table 2 and 3. In group 10–14 the frequency of resorptions was significantly higher than in the controls. The weight per foetus in group 7–11 was significantly lower than in the controls.

The number of ossified coccygeal vertebrae was counted in order to reveal growth retardation in the course of ossification due to thalidomide. The value for the group 7–11 was significantly lower than that for the controls.

In the lower thoracic region, the vertebral bodies were of widely varying appearance ranging from oval or dumb-bell shaped structures to double vertebral centres which were frequently diminished. The anomalies were seen in most of the litters, including the controls, but the frequency was higher and the appearence more obvious in the thalidomide treated groups.

The ossification of the sternum was almost completed in the controls. In both the thalidomide treated groups the number of ossified sternebrae was significantly lower. In a detailed study of the sternum, displacements between the two parts of each ossification centre were observed. They were most frequently seen in the 5th ossification centre but as this is the last to develop it is uncertain whether the displacements are due to an anomaly or incomplete ossification. The 5th ossification centre was therefore not included in the survey of sternebrae anomalies shown in table 3.

Table 2

Effects of thalidomide in rats – parturition.

Dose group	No. of females	Impl/litter $\bar{x} \pm$ S.E.M.	Foetuses/litter $\bar{x} \pm$ S.E.M.	Resorptions			Gross malformations[2]		Weight (g) per foetus $\bar{x} \pm$ S.E.M
				No.	In % of implantations	Coeff.[1] $\bar{}$ ± S.E.M.	No.	In % of foetuses	
I Controls	8	14.0 ± 0.9	13.9 ± 0.9	1	0.9	1.01 ± 0.01	1	0.9	4.2 ± 0.3
II 7–11	10	12.0 ± 0.7	11.3 ± 0.9	7	5.8	1.09 ± 0.04	1	0.9	3.6 ± 0.0*
III 10–14	9	13.9 ± 0.5	13.0 ± 0.7	8	6.4	1.07 ± 0.02	3	2.6	3.9 ± 0.1

Difference from the controls statistically significant ($P < 0.05$).

[1] Coefficiency = $\frac{\text{number of implantations}}{\text{number of alive foetuses}}$

[2] See fig. 1.

Table 3

Effect of thalidomide in rats – skeletal examination.

	Controls ± S.E.M.	Group 7-11 $\bar{x}$ ± S.E.M.	Group 10-14 ± S.E.M.
	No.	No.	No.
Ossified coccygeal vertebrae/foetus	[illegible]5 ± 0.5	1.4 ± 0.1	1.7 ± 0.1
Foetuses/litter with malformed vertebrae	0.8 ± 0.3	3.3 ± 0.7*	[illegible]1 ± 0.6
Malformed vertebrae/foetus	1.0	1.4	1.5
Ossified sternebrae/foetus	5.9 ± 0.0	5.3 ± 0.1	5.5 ± 0.1
Foetuses/litter with malformed sternebrae	1.0 ± 0.4	3.0 ± 0.6	1.4 ± 0.5
Malformed sternebrae/foetus	1.4	1.9	1.8
Frequency of ossified 1st phalanx in the fore paw	95%	9 %	95%

* difference from the controls statistically significant.

Discussion

Although the teratogenic effect of thalidomide is accepted today the pathogenesis of the malformations is still unknown. Factors influencing congenital malformations are numerous. Some comments on the interpretation of the observed malformations in the thoracic vertebrae and the sternum are given below.

The chondrification of the thoracic vertebrae originates at one centre for each vertebral arch and at two for the vertebral body. The ensuing ossification starts at the same centres, but in the vertebral body fusion of the two chondrification centres generally results in only one ossification centre. If this fusion is retarded it results in bicentric or even double ossification centres.

The sternum originates from two lateral free-lying mesenchymal bands which subsequently fuse in a cranio-caudal direction. The ossification proceeds in the same direction except for the xiphoid process, which is ossified earlier. The bicentric ossification centres in the sternum arise by the same processes as those described for the vertebral bodies. The high frequency of ossification disturbances in the thalidomide treated groups and the fact that the two parts of each ossification centre were often displayed, suggests that

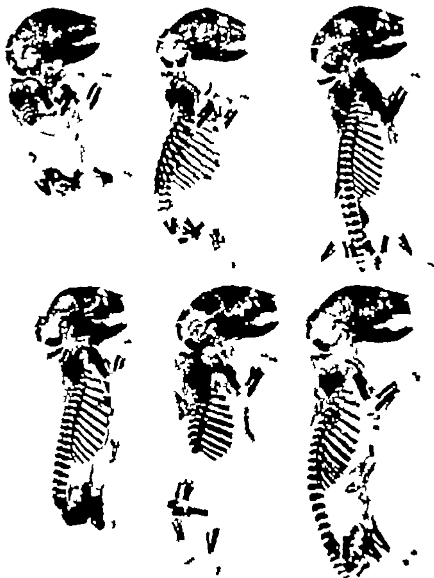


Fig. 1. Rat foetuses with multiple congenital malformations.
To the right a normal foetus.

these anomalies are drug induced. The results were based on an examination on the first four ossification centres since the 5th is not complete at the end of the experiment. However KLEIN OBBINK & DALDERUP (1963) examined rat foetuses after birth and found that the 5th ossification centre was still the one most affected.

It is concluded that the differences between the controls and the thalidomide treated animals indicate a disturbance of differentiation and or growth retardation in the thalidomide treated groups. There was also an increased frequency of resorptions in these groups. The effect of thalidomide was most pronounced in the group treated during days 7–11 of the gestation period.

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From the Department of Toxicology Pharmacia AB, Uppsala, and the Institute of Zoology University of Uppsala, Uppsala, Sweden

Teratological Studies on Thalidomide in Rabbits

By

B. Göran Jonsson

(Received April 13, 1971 Accepted October 14 1971)

Abstract. Pregnant rabbit females, divided into five groups (10–12 in each) were treated as shown below

Treatment, days p.c. (post coitus)	Dose
0–15	5 ml/kg, vehicle (controls)
0– 6	150 mg/kg, thalidomide
7– 9	5 mg/kg, thalidomide
7– 9	150 mg/kg, thalidomide
10–15	150 mg/kg, thalidomide

The substance was homogenised in a 0.5% solution of methylcellulose and was given orally The foetuses were delivered by Caesarean section and were examined for soft tissue and skeletal malformation. The administration of 150 mg thalidomide per kg during days 0–6 or 7–9 of the gestation resulted in a high frequency of resorptions and of congenital malformations. 5 mg/kg given during days 7–9 had no effect either on the frequency of resorption or on malformations. When 150 mg/kg was given from day 10 through day 15 there was no increased frequency of either the number of resorptions or of soft tissue malformations. However the number of skeletal malformations was increased.

Key-words: Thalidomide – abnormalities (drug induced) – abortion.

In 1961 thalidomide was withdrawn from the market because of suspected human teratogenicity Subsequent animal experiments showed, however that similar malformations were difficult to reproduce in different animal species. The first report on congenital malformations in rabbits was published by Somers (1962). Later studies have indicated that the rabbit seems to be the most suitable species for the reproduction of the multiplicity of anomalies found in children. The strain specificity has, however made the results from teratological studies on thalidomide in rabbits somewhat variable.

The aim of the present study was to examine the effects of thalidomide in rabbits when administered during different stages of the gestation period.

Material and Methods

Rabbits of Danish native breed (albinos) weighing 2.7–4.1 kg were used. The day of copulation was considered as day 0 of pregnancy. The mated females were divided into five groups, 10–12 in each, and treated as shown in table 1.

The thalidomide (kindly supplied by Astra Läkemedel AB Södertälje, Sweden) was homogenized in a 0.5% solution of methylcellulose and given orally as a suspension in a volume of 5 ml per kg body-weight. The suspensions were prepared daily.

The experiment was terminated on day 30 *post coitus* (p.c.) when the females were killed with nembutal® (mebumalnatrium NFN) and the foetuses delivered by Caesarean section. The uteri were inspected for implantation sites and resorptions using the technique of SALEWSKI (1964). After weighing, all the young rabbits were examined *post mortem*. The livers, lungs, kidneys and hearts were weighed individually. The animals were then prepared for skeletal examination using the alizarin staining method (CRARY 1962).

During the experiment the females were fed on pellets (Ahl – K – from Ahl-foder Forshaga) hay carrots and water *ad libitum*. Environmental conditions: Temperature 20 ± 2 humidity $40 \pm 10\%$ light ca. 12 hours/day.

Student's t-test was used in the statistical treatment of the results. The tables show the mean value for each group with the standard error of the mean ($\bar{x} \pm$ S.E.M.). Statistically significant difference = $P < 0.05$.

Table 1

Treatment schedule for mated females.

	Group	Treatment, days p.c. (post coitus)	Dose
I	Controls	0–15	5 ml/kg, vehicle
II	0– 6	0– 6	150 mg/kg, thalidomide
III	7– 9	7– 9	5 mg/kg, thalidomide
IV	7– 9	7– 9	150 mg/kg, thalidomide
V	10–15	10–15	150 mg/kg, thalidomide

Results

The results are presented in tables 2–5. The number of implantations per litter showed no significant differences between the groups. However the number of living foetuses per litter was considerably decreased in the groups 0–6 and 7–9 (high) due to the high frequency of resorption in these groups.

There were no significant differences between the groups with regard to litter weight or in the weight of the individual organs.

The size of the central fontanelle and the number of ossified carpal bones, sternebrae and coccygeal vertebrae reflect the stage of the embryonic devel-

Table 2

Effects of thalidomide in rabbits – parturition.

	Group	No. of females	Treatment: Dose mg/kg	Treatment: Duration days p.c.	Impl./litter ± S.E.M.	Alive foetuses/litter ± S.E.M.	No. of dead foetuses	Resorptions: Early[1]	Resorptions: Late[2]	Resorptions: In % of implantations	Resorptions: Coeff.[4] x̄ ± S.E.M.	Malformations	Weight (g) per foetus ± S.E.M
I	Control	10	–	0–15	8.4 ± 0.9	7.8 ± 0.8	–	3	3	7.1	1.08 ± 0.04	See table 4 and 5	49.2 ± 2.4
II	0–6	12	150	0– 6	8.5 ± 0.5	6.1 ± 0.6	1	26	2	27.5	1.70 ± 0.40		45.0 ± 1.8
III	7–9 (low)	10	5	7 9	8.7 ± 0.6	8.2 ± 0.4	–	1	4	5 7	1.06 ± 0.05		46.7 ± 2.1
IV	7–9 (high)	12[3]	150	7– 9	7.9 ± 0.5	5.7 ± 0.6	2	12	13	26.3	1.46 ± 0.12*		45.5 ± 1.5
V	10–15	10	150	10–15	7 7 ± 0.6	7.2 ± 0.7	–	2	3	6.5	1.10 ± 0.07		45.2 ± 1.6

* Difference from the controls, statistically significant (P = < 0.05).

1 Impossible to identify the foetus macroscopically

2 Possible to identify the foetus macroscopically

3 One female in this group had a vaginal haemorrhage, rhinitis and conjunctivitis on day 26 p.c. She delivered on day 27 p.c. The results concerning foetus weights, organ weights etc. are therefore not comparable with the other ones in the group and are not included in the tables.

4 Coefficient = $\frac{\text{number of implantations}}{\text{number of live foetuses}}$

Table 3

Effects of thalidomide in rabbits – skeletal examination.

	Group	No. of ossified carpal bones per foetus $\bar{x} \pm$ S.E.M.	No of ossified sternebrae per foetus $\bar{x} \pm$ S.E.M.	No. of ossified coccygeal vertebrae per foetus $\bar{x} \pm$ S.E.M.	Foetuses with additional ribs	Foetuses with wide central fontanelle (> 2 mm)
I	Controls	0.4 ± 0.3	5.4 ± 0.1	15.8 ± 0.2	63%	18%
II	0–6	0.0 ± 0.0	5.7 ± 0.1	15.5 ± 0.1	71%	27%
III	7–9 (low)	0.1 ± 0.1	5.5 ± 0.1	15.6 ± 0.3	54%	32%
IV	7–9 (high)	0.0 ± 0.0	5.6 ± 0.1	16.0 ± 0.2	67%	43%
V	10–15	0.1 ± 0.1	5.7 ± 0.1	14.3 ± 0.2*	54%	15%

Difference from the controls, statistically significant ($P < 0.05$).

Table 4

Incidence of foetuses with congenital malformations.

Affected organs	Dose group I Controls	II 0–6	III 7–9 (low)	IV 7–9 (high)	V 10–15
Soft tissues					
Head		1		9	
Kidney and ureter		2		11	1
Bladder				2	
Liver	4	3		5	1
Gall bladder		1			
Adrenal				1	
Stomach		1		2	
Spleen		1			
Lungs	1	1	1	9	1
Abdominal cavity				1	
Dwarfism		1			
Placenta	1				
Skeletal tissues					
Skull	3	6	8	24	2
Vertebral column				2	
Sternum		4		4	3
Ribs				1	
Extremities		2		9	7

One or both kidneys from most of the foetuses in three litters were slightly nodular

Table 5

Incidence of congenital malformations – summarized.

Dose group	Controls		0–6		7–9 (low)		7–9 (high)		10–15	
	No.	%	No.	%	No.	%	No.	%	No.	%
Litters with soft tissue malformations[1]	0/10		4/12		0/10		11/12		1/10	
Foetuses with soft tissue malformations[1]	0/78	0	5/74	7	0/82	0	25/70	37	1/72	1
Litters with skeletal malformations[2]	1/10		8/12		2/10		9/12		6/10	
Foetuses with skeletal malformations[2]	3/78	4	11/74	15	5/82	6	35/69	51	12/72	17

1 Abnormal number of lobes in the lungs and livers is not noted as malformations in this table because they are considered to be less important.

2 All anomalies of the extremities are placed among the skeletal malformations.

opment and are thus used as a measure of growth retardation. Except for the number of ossified coccygeal vertebrae in the group 10–15 there was no evidence of retardation due to thalidomide (table 3). Soft tissue malformations were seen most frequently in the groups 0–6 and 7–9 (high) while the skeletal malformations were spread over the groups (table 4). In both instances the highest frequency was found in the group 7–9 (high). The malformations were of widely varying type. Externally the limb and head mal formations were most striking while at autopsy various anomalies of the urinary system predominated.

Discussion

The small differences between the groups in the number of implantations per litter indicate that the substance does not prevent the initial phases of implantation. However the embryotoxic effect of thalidomide is confirmed by the high frequency of resorption in the groups 0–6 and 7–9 (high) In this respect, there is also a dose response effect, as there was no increased embryotoxicity in the group 7–9 (low).

Whether the single soft tissue malformations in different organs are drug induced is uncertain. However almost all anomalies in the region of the head and kidneys were found in the group 7–9 (high) There was considerable variation in the type of renal anomaly found. The absence of one kidney is considered as a true agenesis. A pelvic position of the kidneys indicates a disturbance of the kidney ascendance and is regarded as an embryonic feature. The slight nodules of renal tissue in three litters from the group 7–9 (low) are also considered as an embryonic feature.

An increased frequency of skeletal malformations was seen in all the thalidomide treated groups. Many anomalies in the skull were combined with the soft tissue malformations already described. This is valid for all tooth anomalies which result from an early complex disturbance of mesenchyme aggregation of the cranium. It is also true for cranioschisis which is primarily due to agenesis of special chondrification and ossification centres in the roof of the skull.

All limb anomalies were typical thalidomide embryopathies. The origin of arthrogryposis (fig. 1) remains uncertain but there is no concomitant skeletal anomaly The genesis of the pendulous toes (fig. 1) is most curious, the toe being attached to the paw only by skin. By using X rays it was found, however that the phalangeal bones were normally built and ossified.

The increased frequency of foetal resorptions and the high incidence of congenital malformations indicate that thalidomide has a very potent embryotoxic and teratogenic effect when given to rabbits of Danish native breed during different stages of the gestation period.

Although the potential dangers of thalidomide are undisputed today the mechanisms underlying the interference with normal processes of embryonic



Fig. 1. Rabbit foetus with arthrogryposis and pendulous big toe. Mother treated with 150 mg thalidomide per kg daily during days 7–9 post coitus.

development are still unknown. It is not clear if the effects are primary – the substance acting directly on the foetus, or secondary — by an effect on the maternal organism. Both possibilities are plausible since it has been shown experimentally that thalidomide crosses the placental barrier (BECKMAN 1962 FAIGLE *et al.* 1962).

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From the Department of Toxicology Pharmacia AB, Uppsala and the Institute of Zoology University of Uppsala, Uppsala, Sweden

Thalidomide Teratology in Swine A Preparatory Study

By

B. Göran Jonsson

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Abstract. Pregnant sows were given thalidomide, 1 or 100 mg/kg, from day 17 to day 57 of gestation. The substance was given orally (mixed in the food). During the test various haematological, enzymatic and clinical-chemistry studies were made on the sows. The sows were left for normal farrow after which the piglets were autopsied and a skeletal examination was performed. Neither the clinical studies of the sows nor the subsequent examination of the piglets revealed any effects which could be attributed to the medication.

Key words: Thalidomide – nidation – placenta.

The use of swine for toxicological studies has often been recommended. However very few publications on teratological studies in swine have been found in the literature. EARL *et al* (1964) noticed a disturbance of fertility in swine due to thalidomide. MECKENSTOCK (cited from MÜCKTER 1962) reported a marked sedative effect after thalidomide administration and PALLUDAN (1966) observed foetal malformations in the colon heart, palate and urinary system after treating sows with 15–45 mg thalidomide per kg body weight per day during the first trimester

The present work was intended as a preparatory study for teratological investigations in larger animals, and thalidomide was chosen because of its teratogenic properties in man. During the examination special clinical investigations were performed on the sows.

Material and Methods

Four sows of Swedish native breed, weighing approximately 100 kg, were used. All sows had previously had several litters. Two of them were given thalidomide (kindly supplied by Astra Läkemedel AB Södertälje), 1 and 100 mg/kg respectively from day 17 to day 57 of the gestation period. The substance was given orally (mixed in the food). The control sows received no treatment.

Blood samples for the clinical investigations were taken from the ear veins before the start of thalidomide treatment (17th day of pregnancy), on the 22nd day and on the 44th day (blood samples were taken before drug administration on these days). The following determinations were made: total leucocytes, haemoglobin, total proteins, glucose, GOT (glutamic-oxalacetic transaminase), GPT (glutamic-pyruvic transaminase), ICD (isocitric dehydrogenase), creatinine, alkaline phosphatase and cholesterol.

The sows were allowed to deliver normally. The piglets were then weighed and inspected for external malformations. Half the litters from the thalidomide treated sows were killed by chloroform inhalation and a post-mortem examination was performed. The remaining piglets were left with their mothers for 14 days to observe viability and growth. They were then killed and examined at post-mortem. After the autopsy the piglets were prepared for skeletal examination, using the alizarine staining technique. All control piglets were allowed to survive.

Results

During the pregnancy no visible effects of the thalidomide treatment of the sows were observed.

The clinical investigation in the sows revealed no large differences between the treated and untreated animals.

Both the control sows delivered on the 115th day of pregnancy, the low dosed sow on the 116th day and the high dosed one on the 114th day. The number of foetuses per litter was in the controls 14 and 11, in the low dosed sow 14 and in the high dosed one 10 (in four earlier pregnancies this sow farrowed 13–14 piglets per litter).

The mean weight per piglet in the controls was 1.31 and 1.56 kg respectively, in the low dosed litter 0.99 kg and in the high dosed 1.62 kg. In the latter case the individual weight varied considerably – extreme values being 2.15 and 1.00 kg.

No gross malformations were found at autopsy. The only noteworthy findings were double or bifurcated renal veins in several piglets. Whether this occurs normally in swine is unknown but it is probably not a drug-induced anomaly. On account of these negative results, the killing and autopsy of all control piglets was considered to be unnecessary.

Skeletal examination revealed minor sternal malformations in three piglets (one in the low dosed and two in the high-dosed sow). In 14 of the 24 piglets the fusion in one or several ossification centres of the sternum was incomplete.

Discussion

The time and duration of the thalidomide treatment was chosen with reference to the time of implantation and placentation in swine. The placenta

is morphologically characterised as diffuse and histologically as epithelio-chorial. These two properties and the fact that the uterine mucosa is of a non-deciduate type make the attachment of the blastocyst very loose and the course of implantation lengthy (AMOROSO 1964). The foetuses are not firmly implanted until the 25th day and before this time the risk of abortion is in-increased. Accordingly the thalidomide treatment should start as late as possible but by the 25th day embryonic development is too advanced. Treatment was therefore started on day 17 p.c. At this time the foetus is in the "limb bud-stage" (AREY 1965) and from a development point of view is approximately comparable to a human foetus of 27 days (It is known that thalidomide malformations in man are induced approximately between the 27th and 54th day of pregnancy.) The sows were then treated until the middle of pregnancy day 57 (normal length of pregnancy is 114 days). By this time, the foetus has passed all the "sensitive stages" in development, and malformations are usually not induced later.

In conclusion this study on thalidomide in swine revealed only small differences from the control material with regard to the clinical investigation in the sows as well as to the examination of the piglets. Nevertheless swine seem to be a useful species for this type of study. Their size facilitates sequential clinical investigation in the females and convenient autopsies in the piglets. Attention must, however, be drawn to the large differences in implantation and placentation between swine and for instance rodents, lagomorphs and man.

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Department of Toxicology Pharmacia AB, Uppsala and Institute of Zoology
University of Uppsala, Uppsala, Sweden

Effects of Thalidomide on the Embryonic Development of the Axolotl (Ambystoma Mexicanum)

By

B. Göran Jansson

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Abstract. Axolotl larvae were exposed to thalidomide in concentrations of 0.004 0.04 or 0.2 mg/ml nutrient solution during the following stages (Harrison's stage series) of embryonic development: Series 1 Stage 0–38 or 0–45 Test of activity Series 2: Stage 2–11, Study of the first cell cleavages. Series 3. Stage 3–37 Study of the growth rate. Series 4 Stage 12–46 and Series 5. Stage 25–46, General teratology study Series 6: Stage 37–46, Study of limb differentiation. The results revealed a reduction of activity There was no visible effect of the drug on the first cell cleavages, up to gastrulation, nor on the growth rate. A high frequency of larvae in the thalidomide treated groups showed generalized oedema during different stages of development. The skin in the region of the gills, kidneys and fore limbs was sometimes rough and showed characteristic tremor

Key-words: Thalidomide – oedema – tremor

In addition to the large number of teratological studies on thalidomide in mammals some experiments of quite another type have been described. Bobzy (1963) studied the effect of thalidomide on early cell differentiation of marine red algae (Callithamnion tetricus, Ag) and observed growth inhibition and malformations. Frank *et al.* (1963) found that thalidomide caused a general inhibition of growth in different protozoon cultures.

The aim of this work was to study the effects of thalidomide on the embryonic development of amphibian larvae.

Material and Methods

Eggs from axolotl (Ambystoma mexicanum, Cope) were used. Up to stage 35 (Harrison's stage series) the larvae were reared in nutrient solution (Holtfreter 1931) of half the concentration assigned. After stage 35 tap water was used. During the experiments the thalidomide (kindly supplied by Astra Läkemedel AB, Södertälje) was dissolved or homogenized in the media (the solubility of thalidomide is 0.04 mg/ml in both media). Six different test series were performed.

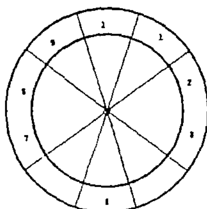


Fig. 1. Glass bowl for activity tests.

Series 1 Test of activity

The method has previously been described by DETWILER (1948) Into a glass bowl 2 cm high and 10 cm in diameter a glass ring was placed of the same height and 8 cm in diameter The bowl was placed on a card divided into 10 sectors (fig. 1).

The larvae were placed individually in the circular channel By slight stimulation with a thin hair loop threaded around the tail, the larvae were induced to swim and the number of sectors passed was noted. Each larva was activated 25 times during the test. By testing a sufficiently large number of larvae for each thalidomide concentration, the effect of the substance on the activity of the animals could be established. Two experiments were made – one with larvae at stage 38 (Coghill's "early swimming stage") the other at stage 45 (Coghill's "early feeding stage") (COGHILL 1929). Each experiment consisted of four groups of larvae which had been reared in thalidomide solutions or suspensions in the following concentrations: a) 0 (controls), b) 0.004, c) 0.04 and d) 0.2 mg per ml. In the first experiment (stage 38) each group contained 12–13 larvae, in the second (stage 45) 17 25. The number of heart contractions per minute was recorded at stage 45

Series 2 Thalidomid study from stage 2 to stage 11 (without vitelline membrane).

The aim of this experiment was to study the effect on the larvae of exposure to thalidomide from the 2-cell stage up to gastrulation, i.e. about stage 11 (possible growth inhibition and/or abnormal development) The test was not started with non-divided eggs since these may be of varying age and development. The 2-cell stage, however is a short and easily defined period.

In order to increase permeability all the membranes, including the vitelline membrane, were removed. This was chieved by putting the non-divided eggs into a dilute solution for some hours. Thus the water diffused through the vitelline membrane which could then be easily removed. During the test the eggs were placed in a cold-storage room (+ 12°) which delayed the development to stage 11 by several days. The experiment was stopped at stage 11 since the eggs do not gastrulate normally when the vitelline membrane is removed (the eggs exogastrulate).

The tests were performed in glass bowls. In order to prevent the larvae from adhering to the glass, a thin gauze was placed in the bottom of the bowl. Four groups (controls, 0.004 0.04 and 0.2 mg thalidomide per ml nutrient solution), each consisting of 20 larvae, were examined.

Series 3 Thalidomide study from stage 3 to stage 37

The effect of thalidomide was studied from the 4-cell stage up to about stage 37 (with the vitelline membrane intact). Three groups with 55 larvae in each were tested – controls, 0.004 and 0.2 mg thalidomide per ml nutrient solution. The larvae were ex amined and stage-determined once per day The experiment was concluded at about stage 37 as the larvae then enter a sensitive period of embryonic development associated with a high mortality Furthermore around stage 36–38 the larvae were often afflicted by fungus disease, which was difficult to prevent.

Series 4 Thalidomide study f om stage 12 to stage 46.

The mortality during gastrulation under normal circumstances may be high, even if the vitelline membrane is intact. The exposure to thalidomide was therefore started immediately after gastrulation, at stage 12, and continued until the development of the fore limbs, stage 46. The test was intended as a general examination of the teratogenic effect of thalidomide. The same four concentrations as in series 1 and 2 were used, each group consisting of 18 larvae.

Series 5 Thalidomide study from stage 25 to stage 46

A similar experiment to series 4 was performed except that it was started at stage 25. The same four concentrations of thalidomide were used with 30 larvae in each group.

Series 6. Thalidomide study from stage 37 t stage 46.

The im of the experiment was to study the effect of thalidomide on the differentiation of the fore limbs. The same four concentrations as in the preceding series were used with 20 larvae in each group.

Results

Series 1 Test of activity

The number of sectors passed per larva after 25 activations is shown in table 1 (mean value ± S.E.M.).

Table 1

	Group	No. of larvae	Sectors passed per larva
Stage 38			
	Controls	13	24 0 ± 4 4
	0.004 mg/ml	13	11.6 ± 2.0*
	0.040	12	6.7 ± 1.5*
	0.200	12	19.9 ± 1.9
Stage 45			
	Controls	17	15 6 ± .4
	0.004 mg/ml	20	8.8 ± 1.5*
	0 040	25	11.1 ± 1.5
	0.200	17	14.3 ± 2.5

Difference from the controls statistically significant.

Table 1

Mouse ileum *in vitro*

Compounds	EC 50 (µg/ml)	Relative activity
TRB	11	1
OPR	1.2	9
KWD 2026	0.06	180
IPR	0.009	1100

The EC 50-values calculated from the results obtained in the *in vitro* studies (compare fig. 1). The relative activity (degree of relaxation) is referred to TRB as unit.

Table 1 shows the activity calculated as µg/ml giving a 50 per cent relaxation (EC 50) as well as the relative activity of the compounds, referred to TRB. From the data obtained, it can be clearly concluded that the relaxing effect is of the order of TRB < OPR < KWD 2026 < IPR. Fig. 2 demonstrates the effects of doses with a multiple of 10 between each effect and approximately in the range of the EC 50.

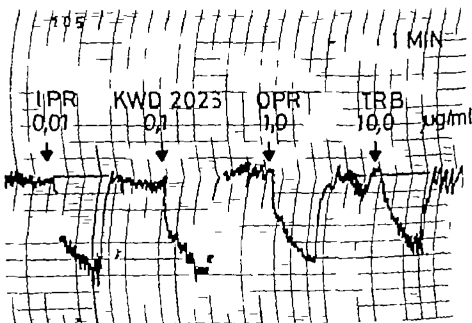


Fig. 2. Mouse ileum *in vitro*. A registration obtained after a dose sequence X 10 is presented. The doses are approximately equi-effective and produce about 30 per cent relaxation.

A concentration of 0 1 μg/ml of alprenolol in the Krebs solution completely inhibited the relaxing effect of the compounds administered in an equieffective dose range around the EC 50

In vivo.

A. *Intraperitoneal administration.*

Among the doses of compounds selected, the smallest (0.8 mg/kg) was given so as not to separate the response of the test animals from that of the saline-treated control groups. The highest dose administered was calculated from the intraperitoneal LD 50-value in mice. About one third of this value was selected as the maximum dose.

The intestines were examined 1 hour and in some series 2 hours after the charcoal administration. Fig. 3 illustrates the results of experiments including the four substances given in the same dose levels. On the ordinate the distance that the charcoal moved in 1 hour is indicated. The whole intestinal length from the pylorus to the anus corresponds to 100 per cent. The doses administered are indicated on the abscissa. Each point in the diagram represents 10 animals. In the saline treated control groups, comprising 120 animals, the charcoal had passed through 84 (S.E.M. ± 2) per cent of the intestinal length in 1 hour To separate test animals from control animals by the use of TRB a dose higher than 25 mg/kg was necessary but OPR at this dose level displayed inhibiting effects. Otherwise these two drugs tended to act in a similar manner as compared to KWD 2026 and IPR. Fig. 3 also shows the dose response with regard



Fig. 3. Effect on intestinal motility in mice after intraperitoneal administration of TRB (●), OPR (○), KWD 2026 (▲), and IPR (△). The compounds are injected 30 minutes before an oral administration of a charcoal suspension. The doses of compounds used are indicated on the abscissa. On the ordinate, the distance propelled 1 1 hour is given in per cent of the whole intestinal length. In control groups (120 animals) given 0.8 per cent NaCl, the distance covered by the carbon black was 84 ± 2 per cent of the intestinal length. Each point in the diagram represents 10 animals and the vertical bars the standard error of the mean.

to the inhibiting effect of the two last-mentioned compounds, with an appreciable effect at 8 mg/kg. However a difference in activity between the two drugs in the dose range used seems difficult to establish. In an effort to trace the difference in effect between the compounds used, an analysis by means of the all-or-none criterion for carbon black in the caeca was also used. This method of evaluating the inhibitory activity requires a propulsion time of 2 hours for the marking substance to give *a distinct estimate. Fig. 4 shows the results from such a study* Compared to fig. 3 the principal relations in effect between the four substances used seem to be the same and independent of the method of evaluation. In order to establish a statistical significance of the difference in effect between TRB and OPR, these drugs were given to two separate groups, each group consisting of 30 mice. The dose was 80 mg/kg. The number of mice with charcoal-free caeca was compared to the less inhibited animals with black caeca. After receiving TRB charcoal appeared in the caeca in 20/30 of the mice but after OPR only in 7/30 The two groups were compared statistically (χ^2) and the value $P < 0.001$ indicating a less inhibiting activity of TRB was obtained. In order to investigate the mechanism involved in the relaxing effect of β-adrenergic stimulants, a dose of 5 mg/kg of alprenolol was given subcutaneously before the intraperitoneal administration of the drugs or saline. Control animals

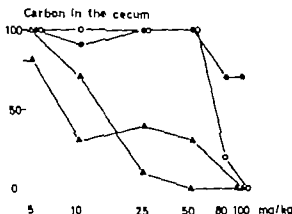


Fig. 4. The figure shows the result obtained from a study with intraperitoneal injection of TRB (●) OPR (○), KWD 2026 (▲), and IPR (△). Thirty minutes after the injection, a charcoal suspension was supplied by stomach tube. Two hours after the charcoal administration, the appearance of black in the caeca was noted. In the figure, the percentage of animals (ordinate) with black caeca can be correlated to the amount of the drugs used (abscissa). In control animals injected with saline, 100 per cent black caeca were observed.

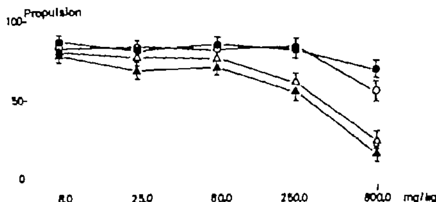


Fig. 5 The figure shows the effect on the intestine of TRB (●), OPR (○), KWD 2026 (▲), and IPR (△) after oral administration 30 minutes before the supply of carbon black by the same route. Control animals (120) receiving saline instead of drugs showed a charcoal propulsion of 82 ± 3 per cent of the intestine between pylorus and anus. Cf fig 3.

pretreated with alprenolol did not differ from the controls treated with saline only. KWD 2026 and IPR were given in about equi-effective doses (10 mg/kg) i.e. comparable to 80 mg/kg of TRB and OPR and resulted in about a 60 / motility in the animals not treated with the β-receptor blocking agent. After pretreatment with the above mentioned amount of alprenolol, no inhibition of peristalsis by the compounds was noted, thus indicating that the amount of alprenolol used produced a complete block.

B *Oral administration*

The same criterion for selection of the dose range, the same type of experiments, and the same principles for evaluation of the results were used as in the study with intraperitoneal administration. Fig 5 shows the results from a study comparable to the one presented in fig. 3 and according to the criterion for the dose range, a 10 times higher dose level was used. The time from charcoal administration until the animals were killed was 1 hour. In the saline treated control groups, run parallel with the test animals, a propulsion of 82 (S.E.M ± 3) per cent was noted. According to the experiments, TRB and OPR can apparently be listed in one group and KWD 2026 and IPR in another without any clear separation within the groups. An evaluation based on the appearance of carbon black in the caeca after a propulsion time of 2 hours showed no change. When 5 mg/kg of alprenolol was administered to animals given equi-effective doses, or 800 mg/kg of TRB and OPR and 250 mg/kg of KWD 2026 and IPR, corresponding to about 60 / propulsion, no in-

fluence whatever was noted from the blocking drug. A dose of 25 mg/kg of alprenolol was necessary to influence the inhibitory activity of the adrenergic compounds used.

Discussion

Sympathomimetic agents are generally believed to induce their relaxing effect on intestinal smooth muscle by stimulating adrenergic α- and β-receptors (AHLQUIST & LEVY 1959, FURCHGOTT 1960, REDDY & MORAN 1968, BÜLBRING & TOMITA 1969). The compounds used in this investigation are classified as adrenergic β-receptor stimulating agents, but the β-receptors can be divided into β_1 and β_2-groups (LANDS *et al.* 1967). Stimulation of the former induces a relaxation of the intestine, but also has an accelerating effect on the heart. The latter relaxes the smooth muscle tension in the respiratory system and peripheral blood-vessels. In the studies of PERSSON & OLSSON (1970) and of PERSSON & JOHNSON (1970) the compound terbutaline has been evaluated as a β_2-receptor stimulating agent with a high selectivity between the activity on smooth muscle in the respiratory tract and on the heart.

In PERSSON & OLSSON'S (1970) investigation, TRB was also compared to OPR and IPR with regard to its relaxing activity on the isolated rabbit duodenum. Moreover, Lands *et al.* (1967) demonstrated a difference in effect between IPR and KWD 2026 by the use of the same preparation. The results from the above mentioned experiments *in vitro* in the rabbit agree well with the data obtained on isolated mouse ileum in the present study, where approximately a dose difference of 10 is registered between the different EC 50-values of the compounds. *In vivo* the relaxing effect of TRB and OPR on the mouse gut can be separated after intraperitoneal administration though this is not the case when the compounds are given orally.

As mentioned above, the four agents used are active on bronchial smooth muscle. It is perhaps of interest to compare the doses giving relaxation of a trachea muscle preparation from guinea-pig (PERSSON & OLSSON 1970) and of the isolated mouse ileum. TRB is about 1000 times more active on the tracheal muscle than on the ileum preparation. OPR is less than 1000 times, and KWD 2026 and IPR both about 10 times more active on the trachea than on the intestine. An approximate comparison between the bronchodilatory activity on non-narcotized guinea-pigs subjected to histamine aerosol (unpublished) and the inhibition of the propulsion in charcoal mice" gives the following result. Doses of about 0.2 and 0.5 mg/kg of TRB or OPR, respectively, and of about 0.05 mg/kg of KWD 2026 or IPR, administered intraperitoneally to guinea-pigs, produce a definite broncho-

dilatory effect. To produce an effect on the intestine in mice, however 50–100 times the dose necessary to induce bronchospasmolysis must be used. Moreover the four compounds, administered orally in an amount of about 1 mg/kg, induce bronchospasmolysis in the guinea-pigs, though more than 250 mg/kg of TRB or OPR and more than 100 mg/kg of KWD 2026 or IPR, is necessary to influence the intestine in mice. The relaxation of tracheal smooth muscle is, as mentioned above, mediated by β_2-receptors and the inhibition of the intestine by β_1-receptors. Consequently the dose-effect relation obtained from the two smooth muscle preparations indicates the degree of selectivity of the compounds used and points to TRB as a characteristic β_2-receptor agonist. When comparing the four compounds in the present investigation, the study of the effects *in vivo* in particular indicate that the substances can be divided into two groups. The compounds with OH in the 3–4 position in the benzene ring are listed as being more active in producing relaxation than the substance with OH-in the 3–5 position. However the isolated mouse ileum makes it possible to select the difference within each group of compounds. Between the substances in a group, i.e. OH in the 3–4 and in the 3–5 position, respectively the i-propyl configuration on the nitrogen atom gives the compound a higher degree of relaxing activity than the t-butyl configuration. From the present results, it is evident that of the four compounds tested, the one with the hydroxy group in the 3–5 position and the t-butyl substituted at the nitrogen, i.e. TRB is the substance least likely to cause any disturbance in gastrointestinal motility

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From the Research Laboratories, AB Draco, Research and Development Department, Fack, S-221 01 Lund, Sweden

An Evaluation of the Toxicity of Adrenaline, Isoprenaline, and Terbutaline after Pretreatment with Isoprenaline and Terbutaline

By

O. A. Torsten Olsson

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Abstract. The toxic effects of adrenaline, isoprenaline, and terbutaline have been determined in mice after pretreatment of the animals with isoprenaline, terbutaline, and saline solution (control). Pretreatment with isoprenaline and terbutaline was without any significant effect on the mortality (LD 50) due to isoprenaline and terbutaline. The sensitivity to adrenaline, however was considerably increased after pretreatment with isoprenaline but only slightly after terbutaline.

Key words: Terbutaline – isoprenaline – adrenaline – drug interaction toxic effects – mice.

The new bronchospasmolytic drug terbutaline (TRB) (bricanyl®), as well as isoprenaline (IPR) and adrenaline (ADR), belong to the sympathomimetic group. However TRB exhibits a high degree of selectivity in its effect as demonstrated by its potency in relaxing bronchial smooth muscle, with only a weak stimulating effect on the heart (Bergman *et al.* 1969 Persson & Olsson 1970 Persson & Johnsson 1970). Toxicity studies can be used for evaluating the effects of drug combinations (Opdyke *et al.* 1970) In the present drug interaction study the toxic effect of TRB was compared with that of IPR and ADR in mice pretreated with IPR and TRB.

Material and Methods

Male mice of NMRI strain, weighing 20–28 g, were used in the study Both before and after treatment, the animals were supplied with water and food *ad libitum* They were housed in plastic cages with a floor area of 18 × 24 cm. Each cage held 10 animals, which were housed together both before and after the treatment. The temperature during the experiment was 22 ± 1 Compounds and saline solution

were administered in a volume of 0.1 ml per 10 g. As a pretreatment, IPR and TRB were administered subcutaneously at the two dose levels i.e. 30 and 90 mg/kg, the highest dose being calculated as about 1/3 × LD50 *subcutaneously*. Thirty minutes after the IPR and TRB pretreatment, ADR, IPR, and TRB were given intraperitoneally in order to determine the median lethal dose. Usually 5 or 6 dose levels in log sequence were used to determine the LD50 of the compound, 10 animals being injected at each level. The mortality rate was determined 1 hour 24 hours, and 5 days after the administration. *Corresponding LD50 values were also calculated* from intraperitoneal injections in the same number of control animals run in parallel as in the tests. As pretreatment the controls were given saline solution.

Compounds used in the study

dl Isoprenaline sulphate (Sigma) l-adrenaline bitartrate (Sigma): *terbutaline sulphate*. 1-(3,5-dihydroxyphenyl)-2-(t-butylamino)-ethanol (AB Draco, Lund, Sweden). Doses given refer to the base form. The compounds were dissolved in 0.8 per cent NaCl containing 0.1 mg/ml ascorbic acid. The saline administered to the control animals also contained this amount of ascorbic acid.

Results

Tables 1 and 2 give the mortality rates (LD50) resulting from the combined treatment. A comparison with the control group shows that the toxicity of IPR is only moderately increased by pretreatment with IPR (table 1). The rate of increase in the toxicity related to time in animals pretreated with saline is also about the same as in animals pretreated with IPR. The toxicity data on ADR from saline-treated control groups as compared with the data obtained after pretreatment with IPR show a great difference. The LD50 after 1 hour for animals pretreated with 30 mg/kg IPR does not differ significantly from that for the corresponding control groups ($P > 0.1$) but the values *after 24 hours, and especially the data* obtained after 5 days, differ significantly from those of the controls ($0.01 > P > 0.001$ and $P < 0.001$ respectively). The effect of the pretreatment on the toxicity of ADR is still more potentiated after 90 mg/kg IPR. Here the LD50 value obtained after 1 hour differs significantly from the value obtained for the control group ($0.01 > P > 0.001$). Not even a pretreatment with 90 mg/kg IPR is apparently sufficient to change the toxic effects of TRB significantly ($P > 0.1$) and no increase in late toxicity is noted for the drug. Table 2 present the results obtained after pretreatment with TRB. The LD50 of IPR and TRB is not influenced by this pretreatment and the LD50 of ADR is only slightly decreased, as compared to control animals run in parallel, after 5 days ($P = 0.05$).

In an attempt to evaluate the toxicity more qualitatively mice treated with IPR only and also with the combination as presented in Table 1 have been sectioned and examined at low magnification. The most obvious

Table 1

The LD50 of ADR, IPR, and TRB determined in mice pretreated with saline (control) or IPR. The mortality rates were recorded after 1 hour 24 hours, and 5 days. The statistical significance of the influence on the LD50 of the IPR and TRB treatment as compared with the control animals is also given in the table. The figures are calculated from the experimental data according to SPEARMAN *et al* 1964.

Pretreatment:	0.8 % NaCl (s.c.)			IPR 30 mg/kg (s.c.)			IPR 90 mg/kg (s.c.)		
Toxicity of.	ADR	IPR	TRB	ADR	IPR	TRB	ADR	IPR	TRB
LD50, mg/kg (i.p.) ± S.E.M. 1 hour	4.9 ± 0.5	368 ± 25	220 ± 32	4.3 ± 0.5 P > 0.1	322 ± 42	205 ± 31	3.[illegible] ± 0.4 P < 0.01 P > 0.001	325 ± 33	174 ± 28
LD50, mg/kg (i.p.) ± S.E.M. 4 hours	4.5 ± 0.4	362 ± 22	215 ± 28	3.0 ± 0.4 P < 0.01 P > 0.001	310 ± 30 P > 0.1	192 ± 20 P > 0.1	1.4 ± 0.2 P < 0.001	315 ± 32 P > 0.1	168 ± 25 P > 0.1
LD50, mg/kg (i.p.) ± S.E.M 5 days	4.5 ± 0.4	360 ± 30	215 ± 28	2.2 ± 0.4 P < 0.001	304 ± 33	190 ± 20	0.8 ± 0.2 P < 0.001	315 ± 32	168 ± 25

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From the Research Laboratories of AB Draco, Research and Development Department, Fack, S-221 01 Lund, Sweden

The Interaction between Theophylline and some Adrenergic α and β-Receptor Agonists Evaluated as Effect on the LD50 of Mice

By

O. A. Torsten Olsson

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Abstract. The toxic effects (LD50) of noradrenaline, adrenaline, isoprenaline and terbutaline have been evaluated in mice pretreated with theophylline (theophyllamine). Compared with saline pretreated animals, the sensitivity to noradrenaline and adrenaline is significantly lowered, but that to isoprenaline and terbutaline remains unchanged.

Key words. Theophyllamine – adrenoceptor agonists – toxic effects – mice.

Of the four sympathomimetic agents tested in this study the compound 1-(3,5-dihydroxyphenyl)-2-(t-butylamino)-ethanol, i.e. terbutaline (TRB) or bricanyl® is a new selective bronchospasmolytic agent (BERGMAN *et al.* 1969), (PERSSON & OLSSON 1970) (PERSSON & JOHNSON 1970) To evaluate the pharmacological effects of TRB studies on the interaction between this compound and other drugs used in, e.g. the treatment of asthma have been performed. Thus, in the present investigation, mice have been pretreated with theophylline (TP) given as theophyllamine, and the interaction between this drug and TRB dl-isoprenaline (IPR), l-adrenaline (ADR), and l-noradrenaline (NA) has been evaluated as the effect on the LD50 of these amines.

Methods

Male mice (strain NMRI, 20–25 g) were used in this study The animals were housed in plastic cages with a floor area of 18 × 24 cm, 10 animals in each cage. The temperature of the room was 22° ± 1 The animals were starved during the night before the test, but were supplied with water *ad libitum*. TP was administered orally by stomach tube (0.5 ml/20 g) and at the same time the control groups received saline (0.9 per cent NaCl) with an adjusted pH, by the same route. The amount of TP used was 100 mg/kg or about 20 % of the LD50 for this drug

Table 1

The LD50 of NA, ADR, IPR, and TRB determined in mice after oral administration of TP

Pretreatment	Saline (p.o.)				Theophylline (100 mg/kg p.o.)			
Toxicity of	NA	ADR	IPR	TRB	NA	ADR	IPR	TRB
LD50 ± S.E.M. 1 hour	29 ±8	10 ±2	332 ±36	205 ±22	190* ±22	158* ±20	302 ±22	196 ±21
LD50 ± S.E.M. 24 hours	28 ±8	6 ±1	320 ±41	205 ±22	121 ±8	96 ±15	280 ±26	196 ±21
LD50 ± S.E.M. 5 days	20 ±7	4 ±1	320 ±41	205 ±22	63 ±8	70* ±15	250 ±26	196 ±21

significantly ($P < 0.001$) different from saline pretreated controls.
The figures in the table and the degree of significance were calculated from the experimental data according to SPEARMAN *et al.* (1964).

after oral administration. The sympathomimetic amines were injected intraperitoneally 15 minutes after the administration of TP or saline. The adrenergic drugs were usually given at 4–6 dose levels, 10 animals being used for each level. The mortality was noted after 1 hour 24 hours, and 5 days. The dose of theophyllamine (theophylline with ethylenediamine) is referred to the amount of theophylline, and the levels of the sympathomimetic amines used refer to the base form.

Results

Table 1 records the data obtained. From the figures of the LD50-values, the protective properties of TP against the agonists possessing an α-receptor stimulation component (or NA and ADR) seem to be conspicuous. The combination of TP with NA and ADR significantly increases the LD50 ($P < 0.001$) of the amines as compared to the control experiments including animals pretreated orally with saline. Pretreatment with TP has no effect, or possibly produces a slight although not significant elevation in the toxicity of the β-receptor stimulating agents IPR and TRB

Discussion

Results from a toxicity test give only a sum of the effects from the compounds tested and consequently it is difficult to interpret a quantitative investigation in a qualitative manner Suggestions for an explanation of the

data obtained in the present study must be hypothetical and the author prefers to give only a brief presentation of some investigations dealing with TP and the sympathomimetics.

In a study of interaction, it is, of course, necessary first to make clear the effects which each of the compounds involved might have in a biological system. Vasodilator effects have been ascribed to TP and its influence on coronary flow has especially been studied (BOYER & GREEN 1941 BAYLAY *et al* 1944 MOKOTOFF & KATZ 1945). However TP has been of little or no value in the management of myocardial infarction or angina pectoris since the drug also produces considerable myocardial stimulation (MELVILLE & LU 1950· SCHMIDT 1951 KLEIN 1969 STRUBELT *et al* 1970) The stimulating activity of TP may be due to the property of the drug to release endogenous catecholamines (WESTFALL & FLEMING 1968 STRUBELT & SIEGERS 1968) Moreover TP is able to inhibit the activity of phosphodiesterase and thereby the enzymatic breakdown of cyclic AMP (BUTCHER & SUTHERLAND 1962). Like other methylated xanthine derivatives, it also exerts an influence on the release of intracellular Ca^{++} (DE GUBAREFF & SLEATOR 1965). Sympathomimetic amines, on the other hand have a wide spectrum of effects which influence a biological system. The activities that determine the result in a study of toxicity are largely related to the potent effect of the drug on the cardiovascular system the cardiac system as well as the smooth muscle in the vessels are greatly affected (WENZEL 1967).

In this study the LD50 of the α-stimulating agents NA and ADR is significantly increased by the TP treatment. At first, it is tempting to refer the effect of TP to the vasodilator activity or this drug since an α-stimulation has the reverse effect. This view is supported by a study of OPDYKE *et al.* 1970 in which the coronary dilator drug glyceryltrinitrate increased the LD50 of ADR. However RICHARDS 1941 and GARDNER *et al.* 1950 found that the toxic effect of the vasopressor and α-stimulating drug ephedrine was greatly increased by TP while on left atrium preparations, the stimulating effects of NA (RALL & WEST 1963) were potentiated by TP However inhibitory effects of TP have also been reported. HESS *et al.* 1963 described a decrease in the contractility in isolated rat hearts after combined treatment with TP and ADR, which occurred despite an increase in phosphorylase. In an investigation in 1969 MCNEILL *et al.* also reported that doses of TP enhancing phosphorylase after NA, decreased the inotropic response of the heart in open-chest rats. BOWMAN & HALL (1970), believe that TP in higher concentration exhibits inhibitory actions on the response of e.g. ADR. TP seems to influence a mechanism other than the phosphodiesterase system, and a disturbance of the Ca^{++} flux is perhaps of importance in this case.

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From The Institute of Pharmacology University of Oslo, Blindern, Oslo 3, Norway

Reduction in ^{42}K Efflux from Rat Atria by Promazine and Thioridazine

By

Knud Landmark,[1] Jon F W Haffner[2] and Johannes Setekleiv

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Abstract: Rat left atria were suspended for one hour in Ringer solution containing approximately 6 µcl/ml ^{42}K. They were then transferred to a constant flow apparatus and the ^{42}K-efflux was determined by measuring the radioactivity in the effluent. The mechanical activity was recorded simultaneously The atria were driven by electrical stimulation at 180 beats per min. In normal Ringer solution a constant decrease in potassium efflux, assumed to reflect the transfer of ^{42}K through the cell membranes, was obtained after about 15 min. The addition of promazine 5×10^{-6}M to 5×10^{-5}M and thioridazine 10^{-6}M to 5×10^{-6}M to the Ringer solution caused a dose dependent reduction in ^{42}K-efflux and in the amplitude of the atrial contractions. A reduction in the extracellular potassium concentration decreased, while an increase of the extracellular potassium concentration increased the ^{42}K-efflux. The per cent reduction in ^{42}K-efflux *caused by promazine 5×10^{-5}M was almost the same under these experimental* conditions, whereas the depressant action of the drug on the atrial contractile force increased with increasing concentrations of potassium. No direct proportionality between the reduction in ^{42}K-efflux and the reduction in contractile force caused by promazine was found. It is concluded that the decrease in ^{42}K-efflux caused by the drug is due to a reduced membrane permeability for this ion, and that the decrease in contractile force can be dissociated from this effect.

Key-words: Phenothiazine derivatives – rat atria – ^{42}K-efflux – contractile force.

It has been suggested that the cardiac effects of phenothiazine derivatives *are basically similar to those of quinidine* and other antifibrillatory drugs (LANGSLET 1970 LANDMARK 1971b) Quinidine and high concentrations of β-adrenergic receptor blocking agents reduce the efflux of both potassium (HOLLAND 1957 HOLLAND & BRIGGS 1959 COHN & WOOD 1960; KLEIN *et al* 1960; RAIN & REUTER 1966) and rubidium, an ion which can be conveniently used as a tracer for movements of K ions (van ZWIETEN 1969) from cardiac muscle. Thus it seems as if there may be a correlation between antiarrhythmic activity and the ability to reduce K -efflux. The

1 Research Fellow The Norwegian Council on Cardiovascular Diseases.

2 Research Fellow The Norwegian Council for Science and Humanities.

phenothiazine derivatives promazine, thioridazine and chlorpromazine have been shown to decrease the K loss from isolated perfused rat hearts (LANGSLET 1970, LANDMARK 1971a). The K[illegible]-loss in these experiments was, however, determined indirectly from the increase of the K concentration in the perfusate. The present investigation was undertaken with the aim of studying the efflux of radioactive potassium more directly. Isolated rat atria stimulated at a constant frequency were used, and the ^{42}K-efflux was measured before and after exposure to promazine and thioridazine. The effect of promazine on ^{42}K-efflux was compared with the effect on contractile force.

The extracellular concentration of potassium, [K]o is known to influence the cardiac response to quinidine (ARMITAGE 1957, HOLLAND 1957, WATANABE *et al.* 1963, BRANDFONBRENER *et al.* 1966, WATANABE & DREIFUS 1967), and experiments carried out in our laboratory have shown that variations in [K]o also influence the effects of promazine and thioridazine on the automaticity, contractile force, refractoriness and excitability of rat atrial muscle (LANDMARK 1972). In order to determine whether the alteration in K -efflux produced by promazine is dependent on the [K]o, the effects of promazine at varying [K]o have also been studied.

Methods

Female, albino Wistar rats (about 250 g) were used. The hearts were excised under ether anaesthesia, the left atria were removed by dissection, tied to a holder and suspended in a water-jacketed organ bath containing 30 ml radioactive (^{42}K) modified Ringer solution at 32° bubbled with 95% O_2 and 5% CO_2. The Ringer solution had the following composition (ions in meq./l): sodium 143.4, potassium 5.3, calcium 5.1, magnesium 2.3, chloride 126.4, phosphate ($H_2PO_4^-$) 2.4, bicarbonate 25, sulphate 2.3 and glucose 1.8 mg/ml. The specific activity of the solution was approximately 6 µCi/ml.

After one hour incubation in this solution, the atria were transferred to a constant flow apparatus (BRATENG 1967) which was perfused at a rate of 2–3 ml/min. with non-radioactive Ringer solution of the same composition and temperature as the incubation fluid. The transfer and mounting took approximately 2 min. A preload of 400 mg was put on the preparations, and the atrial contractions were recorded isometrically with Grass force-displacement transducer (FT03C) connected to a Grass polygraph (Model 7WC12PA). The effluent solution containing ^{42}K from the atria was collected in plastic test-tubes which were changed every second min. by an automatic fraction collector (LKB Ultrorac 7000). The radioactivity of each sample was determined in a Packard Auto-gamma spectrometer Model 3001.

The atria were stimulated electrically through platinum electrodes – one of which was in direct contact with the atrium, the other was submerged in the solution – with 6 V square wave pulses of 0.5 msec. duration (suprathreshold stimulation) at a frequency of 180 per min. After an initial 32 min. equilibration and control period, the drugs were added to the Ringer solution in amounts giving final concentrations from $5 \times 10^{[illegible]}$ M to $5 \times 10^{[illegible]}$ M.

The atria were exposed to this test solution for a further 32 min. period. In the experiments where the effects of varying [K]o were investigated, the initial K content of 5.3 meq./l was changed to either 10.6 meq./l (high [K]o) or 1.325 meq./l (low [K]o) after the initial equilibration period. No osmotic adjustments were made when [K]o was altered because the osmotic variations induced were considered to be insignificant.

After the experiments, the radioactivity remaining in the atria was determined and the atria were dried at 60 for 4 hrs before the dry weight was determined.

Calculations.

The content of ^{42}K in the preparation throughout the experiment was calculated by adding the radioactivity of the samples of effluent to the radioactivity which remained in the preparation at the end of the experiment. The fraction lost at any time during the experiment was determined by dividing the amount of tracer lost per min. by the amount of tracer which the preparation contained at that time.

When the effect of the drugs were studied, the efflux of ^{42}K was calculated in counts per mg dry weight min. 1. In order to compare the results, the total number of counts obtained over the last 8 min. of the equilibration period in each experiment was called 100 per cent and used as reference for the values found in the subsequent test period which was subdivided into periods of 8 min. duration.

Drugs used.

Promazine HCl (Norfarma) and thioridazine HCl (Sandoz) were dissolved on the day of the experiments in de-ionized water and kept in the dark at 4 Radioactive potassium (^{42}K) was supplied by Institutt for Atomenergi, Kjeller

Results

The effects of promazine and thioridazine on ^{42}K-efflux

In fig. 1 the mean fraction of ^{42}K lost per min. from atria not exposed to phenothiazines, is plotted semi-logarithmically against time. After about 15 min. wash-out, the curves fell slowly and linearly indicating that these values represent the transfer of ^{42}K through the cell membrane. An increase in [K]o increased the fraction of ^{42}K lost per min., while a decrease in [K]o had the opposite effect (fig. 1) These changes were complete within about 6 min. when a new steady level was reached.

When the K concentration of the Ringer solution remained unchanged throughout the experiments, the addition of promazine decreased the ^{42}K efflux from the rat atria (fig. 2 A) Promazine 5 × 10 ^{6}M (not shown in the figure) and 10 ^{5}M had only a slight inhibitory effect on the ^{42}K-efflux, while 5 × 10 ^{5}M markedly reduced the efflux. Thioridazine 10^{-4}M caused a small decrease in the ^{42}K-efflux, while the effect of 5 × 10^{-4} M was pronounced (fig. 2 B) The per cent reduction in ^{42}K-efflux produced by various concentrations of the two drugs is shown in fig. 3 On a molar basis promazine decreases ^{42}K-efflux more than thioridazine.

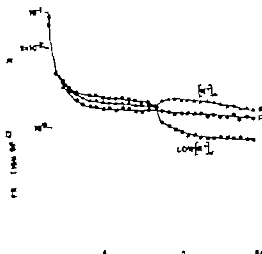


Fig. 1 Semilogarithmic plot of fraction of ⁴²K lost per min. during wash-out from left rat atria which had been labelled with ⁴²K. I some experiments, the initial K concentration of the Ringer solution (5.3 meq./l) was changed to either 10.6 meq./l (△) or 1.325 meq./l (□) after a period of 32 min. ○ = [K]o kept constant (5.3 meq./l) throughout the experiments. In brackets number of experiments.

When added coincidently with alterations in [K]o promazine 5×10^{-6} M reduced ⁴²K-efflux (fig. 4 A and B) The per cent reduction in ⁴²K-efflux caused by 5 × 10 ⁶M promazine was found to be roughly the same towards the end of the test period at all concentrations of [K]o used (fig. 5).

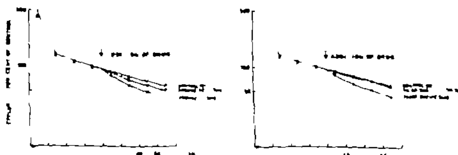


Fig 2. Reduction in ⁴²K-efflux from left rat atria caused by promazine (A) and thioridazine (B). The total number of counts recorded during the last 8 min. of the equilibration period in each experiment was called 100 per cent and used as reference for the values found in the subsequent test period, which was subdivided into periods of 8 min. duration. In brackets number of experiments.

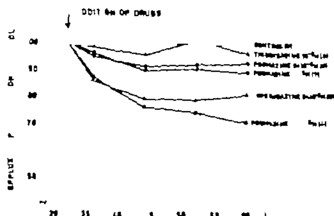


Fig. 3. Reduction in ^{42}K-efflux caused by promazine and thioridazine at different time intervals throughout the test period. The mean efflux values obtained from 3 control atria were called 100 per cent and used as reference for the efflux values obtained from atria treated with the drugs. In brackets number of experiments.

The effect of promazine and thioridazine on the contractile force

The addition of promazine and thioridazine to the Ringer solution caused a dose dependent decrease in contractile force. The reduction produced by promazine 5×10^{-6}M and 10^{-5}M was preceded by a slight augmentation of the contractions of 3–8 min. duration. While the decrease in contractile force induced by the lower concentrations of either drug was small, 5×10^{-5}M of promazine and thioridazine caused a pronounced and progressive decline in contractile force (fig. 6).

One atrium out of four which had been exposed to promazine 5×10^{-5}M

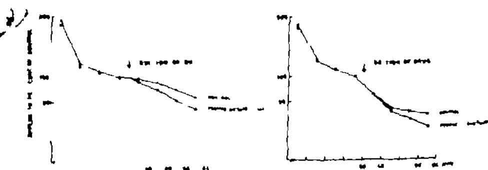


Fig. 4. Reduction in ^{42}K-efflux from left atria caused by 5×10^{-6}M promazine. The drug was added to the Ringer solution after an equilibration period of 32 min. At the same time, $[K^+]_o$ was changed from 5.3 meq./l to 10.6 meq/l (A) or 1.325 meq/l (B). Calculations as in fig. 2. In brackets number of experiments.

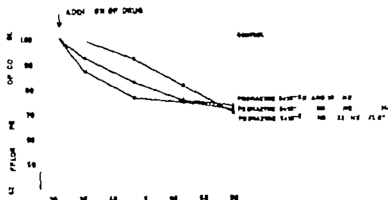


Fig. 5. Reduction in ^{42}K-efflux caused by 5×10^{-6} M promazine at different time intervals throughout the test period at varying [K]o The mean efflux values obtained from 6 control atria (low [K]o) 3 control atria ([K]o kept constant) and 5 control atria (high [K]o) respectively were called 100 per cent and used as reference for the efflux values obtained from atria treated with promazine. In brackets number of experiments.

failed to follow the electrical stimulation towards the end of the test period.

When [K]o was decreased or increased, a slight reduction in contractile force occurred. The addition of promazine 5×10^{-6} M which coincided with the increase in [K]o from 5.3 meq /l to 10 6 meq /l, caused a pronounced and progressive decline in contractile force, and after approximately 15 min., three out of four preparations failed to follow the electrical stimulation

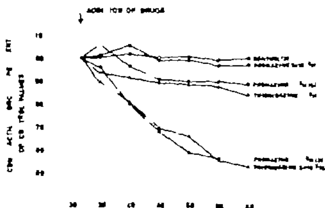


Fig. 6. Changes in contractile force caused by promazine and thioridazine. The values obtained after 32 min. equilibration period in each experiment were called 100 per cent and used as reference for the values found in the subsequent test period. In brackets number of experiments.

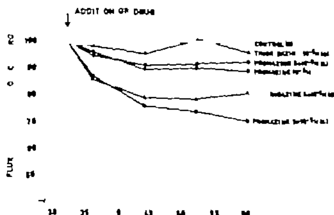


Fig. 3 Reduction in 42K-efflux caused by promazine and thioridazine at different time intervals throughout the test period. The mean efflux values obtained from 3 control atria were called 100 per cent and used as reference for the efflux values obtained from atria treated with the drugs. In brackets number of experiments.

The effect of promazine and thioridazine on the contractile force

The addition of promazine and thioridazine to the Ringer solution caused a dose dependent decrease in contractile force. The reduction produced by promazine 5×10^{-6}M and 10^{-5}M was preceded by a slight augmentation of the contractions of 3–8 min. duration. While the decrease in contractile force induced by the lower concentrations of either drug was small, 5×10^{-5}M of promazine and thioridazine caused a pronounced and progressive decline in contractile force (fig. 6)

One atrium out of four which had been exposed to promazine 5×10^{-5}M

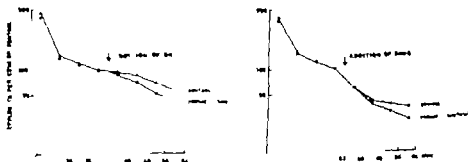


Fig. 4 Reduction in 42K-efflux from left atria caused by 5×10^{-5}M promazine. The drug was added to the Ringer solution after an equilibration period of 32 min. At the same time, [K]o was changed from 5.3 meq./l to 10.6 meq./l (A) or 1.325 meq./l (B). Calculations as in fig. 2. In brackets number of experiments.

Relationship between the effect of promazine on ^{42}K-efflux and on contractile force.

Alterations in [K]o were found to have marked effects on ^{42}K-efflux, but hardly any influence on the contractile force in electrically stimulated rat atria. The per cent decrease in ^{42}K-efflux caused by promazine was almost independent of [K]o whereas the effect of the drug on the contractile force was greatly altered by changes in [K]o Other differences between the effect of promazine on ^{42}K-efflux and on the contractile force were also obtained the addition of promazine caused an immediate reduction in efflux, while the reduction in the amplitude of the contractions was delayed, and in some experiments this was preceded by an augmentation. The magnitude of the effects was also different. The greatest decrease in ^{42}K-efflux produced by promazine was about 30 per cent, whereas the reduction in contractile force was approximately 60 per cent before the preparations failed to follow the electrical stimulation. In order to compare the effects of promazine on ^{42}K-efflux and contractile force, we have calculated the ratio between ^{42}K-efflux and contractile force expressed in per cent of the control values at different [K]o values throughout the test period (fig. 8) Values below 1 0 in fig. 8 indicate that the drug affects the ^{42}K-efflux more than the contractile force, while values above 1 0 show the opposite condition. Immediately after addition of the drug, values below 1.0 were obtained. This was partly due to a more rapid onset of the effect on ^{42}K-efflux than on the contractile force, and partly due to an initial augmentation of the contractions. Later in the test period, the effect on contractile force exceeded that on the ^{42}K-efflux and values above 1.0 were obtained, except when the two lowest concentrations of promazine were added at 5.3 meq / l. When [K]o was increased, the curve shifted towards the left indicating a greater and more rapid effect on contractile force than on ^{42}K-efflux.

Discussion

The present experiments show that promazine and thioridazine cause a dose dependent decrease in ^{42}K-efflux from electrically stimulated rat atria. Promazine is the more potent compound of the two drugs when compared on a molar basis. These results are in accordance with previous observations (LANDMARK 1971a).

Since K -efflux is believed to be a passive process, the results indicate that the two phenothiazine derivatives change the membrane permeability for K This effect of phenothiazines on passive ion flux through cell membranes is, however not restricted to K ions in cardiac cells. CHRISTENSEN *et al.* (1958) found that chlorpromazine inhibited the entry of radioactive

Na into rat brain *in vivo* an observation confirmed by CHAN & QUASTEL (1970) in stimulated rat brain cortex slices. Inhibition of Ca influx into isolated frog muscles caused by chlorpromazine has also been described (BALZER & HELLENBRECHT 1969) BURTON *et al* (1967) found that the hearts of rats receiving intraperitoneal injections of thioridazine had increased K but decreased Na and water contents.

It has been demonstrated that the initiation of atrial flutter and fibrillation and of ventricular fibrillation in isolated cardiac muscle is associated with a marked, but transient increase in Na influx and K -efflux (KLEIN & HOLLAND 1958 HOLLAND & BRIGGS 1959 BRIGGS & HOLLAND 1961). CONN & LUCHI (1964) have suggested that most arrhythmias are associated with a loss of cell K and gain of Na It has also been assumed that the antiarrhythmic mechanism of quinidine and other anti-fibrillatory agents is in some way related to alterations *in membrane permeability for* K and Na (WATANABE *et al.* 1963 VAN ZWIETEN 1969). The present investigation shows that promazine and thioridazine which are known to possess anti-arrhythmic activity (for review see LANDMARK 1971b) also reduce K -efflux.

K -efflux from cardiac muscle is dependent on [K]o (CARMELIET 1961 GOERKE & PAGE 1965 LANGER & BRADY 1966 BLESA *et al.* 1970) High [K]o increases while low [K]o decreases the K -efflux. Our results are *consistent with these observations. Increasing concentrations of* K have been shown to increase the depressant action of promazine and thioridazine on the excitability of rat atria (LANDMARK 1972) The per cent inhibition in ^{42}K-efflux caused by promazine 5×10^{-5} M is almost the same at different [K]o This means, however that the absolute reduction in ^{42}K-efflux caused by the drug is greatest at high [K]o, and this phenomenon may in some way be related to the potentiation of the phenothiazine-induced changes in *the electrical activity of rat atria exposed to high [K]o. Variations in* [K]o modified the effect of promazine 5×10^{-5} M on contractile force. With increasing [K]o, the depression of the contractions increased. With low [K]o an initial increase in the amplitude of the contractions occurred the subsequent reduction was less than that produced at higher [K]o. An increase in contractile force of electrically stimulated rabbit atria caused by quinidine in the presence of low [K]o has been previously described (HOLLAND 1957). *KENNEDY & WEST (1969) have demonstrated that* quinidine produces a positive or negative inotropic response, according to the nature of the experimental conditions. These authors showed that the negative inotropic effect of high concentrations of the drug was temporarily reversed by increasing the intensity of the stimulus and they suggested that quinidine might exert its positive inotropic action through changes in the availability of intracellular calcium. The same mechanism could explain the temporary augmentation in contractile force due to promazine.

In the present experiments, the reduction in ^{42}K-efflux preceded the decrease in contractile force, and no direct proportionality was found between these two parameters. While the addition of promazine 5×10^{-4}M affected ^{42}K-efflux to a greater extent than contractile force the opposite was the case when 5×10^{-5}M was used. The per cent decrease in contractile force caused by promazine 5×10^{-5}M was enhanced by increasing concentrations of $[K^+]_o$ but the per cent reduction in ^{42}K-efflux remained about the same. The dissociation between the effects on contractile force and on ^{42}K-efflux indicates that different mechanisms are involved in these two actions of the phenothiazines.

Acknowledgements

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From the Department of Biochemistry, Beilinson Hospital, P.O.B. 85, Petach-Tikva, and Department of Physiology and Pharmacology, Medical School, Tel-Aviv University, Tel-aviv, Israel

Investigation of Protein Fractions and Haemolytic Properties of Wasp Venom

By

J. Fischl, J. Ishay, S. Goldberg and S. Gitter

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Abstract. The protein fractions of two wasp species – *Vespa orientalis* and *Paravespula germanica* – were separated by membrane electrophoresis and the relative concentrations estimated by microdensitometry. Most protein fractions found in the first specimen were basic proteins while in the second, considerable amounts of protein fractions migrated towards the anode. However, the main fraction in the second was also electro-positive. It was similar to the main component found in *Vespa orientalis* venom with regard to electrophoretic migration coefficient. The total protein concentration in both kinds of samples were so arranged as to be 500 mg %. The number of fractions detectable was six to seven respectively and differed both quantitatively and qualitatively. Venom obtained by homogenizing the whole venom sac of the insect was also found to be different from the pure venom obtained by the "milking" method and in the following an attempt has been made to explain this difference. The venom possesses a marked haemolytic activity and the haemolytic fractions have also been separated electrophoretically. They are not removed from the venom by dialysis and therefore are either considered to be proteins or to be attached to the proteins. In any case they are high molecular weight substances and thus different from bee venom, which is dialysable. Four haemolytic fractions were found in *Vespa orientalis* venom and their electrophoretic migration is different when the test material is obtained by the "milking" method or by homogenizates of the sac. The haemolytic activity of the pure venom and of the venom extracts were assayed by a method developed by the present authors and are given in detail in the text.

Key-words: Wasp venom – proteins – haemolytic properties – electrophoresis.

Wasp venom is a colourless, viscous liquid with a solid content of 27.3 % of which 76 % are protein. The physical and chemical properties of *Vespa orientalis* venom have already been investigated (H. Joshua, J. Fischl, B. Hendig, J. Ishay & S. Gitter, unpublished results). The venom

is a highly complex biological fluid and is markedly toxic (EDERY & ISHAY 1968), and loses little or none of its potency on lypophilisation or on storage at −20° Neither the extent of lethality in the mouse nor the haemolytic activity of the venom is diminished after dialysis (J ISHAY J FISCHL, S GITTER, unpublished results) It is consequently assumed that most of the toxic principles are high molecular weight substances, or are firmly bound to proteins.

In order to investigate the biological activity of the venom, it is necessary to separate the protein components by the most efficient method. Since the collection of pure venom is extremely difficult, the material available is limited. The most satisfactory method of separation was found by micro-electrophoresis. In order to determine the biological activity of the separated fractions, suitable methods were developed, and the results will be published shortly

The present paper deals with the separation of venom proteins by membrane electrophoresis, and with the haemolytic properties involved. The qualitative and quantitative results are presented.

Methods and Materials

Wasp venom was collected by "milking" the insects, e.g. by applying pressure on the dorsum until the stinger protruded and a micro-drop of the venom appeared in its tip. The venom was removed by touching it with a cooled microslide. When a sufficient quantity of venom had been collected from a large number of wasps, it was lyophilised and then stored in ampoules at −20 Samples from insects injured in the process were discarded. The time for collecting the venom was at the height of social activity – *July-August* – only active "workers" *being* used. Before processing, the venom was dissolved in saline in such an amount as to give a final concentration of 500 mg % of total protein.

Electrophoresis was carried out in a Beckman's "Micro Zone" apparatus, using a 0.05 M barbital buffer of pH 8.6. The current applied was 250 V and the running time, 20 minutes. The amount of each sample was 1 μl, and was obtained by *four* applications by means of a Beckman's applicator For staining, 0.01 % of nigrosin in 3 % sulphosalicylic acid was used. Quantification was achieved by microdensitometry of the pherogram, followed by planimetry of the scan.

Venom sac extract was prepared by homogenization of the complete sacs with saline, removing the tissue débris by centrifugation, and arranging the total protein concentration of the supernatant to 500 mg% with saline.

As an orientation aid to the position of the protein fractions, normal human sera were electrophoresed in parallel with each run. The amount of serum applied was 0.25 μl. Blood agar was prepared by mixing 10 ml of lukewarm agar (2 % in saline) with 1 ml of the donor's blood. The same blood-agar was used for the assay of the haemolytic fractions separated by membrane electrophoresis. *The* method designed by us was to superimpose the membrane folio (porous side down) on blood-agar slides. The haemolytic effect was detected after *60–120* min., both in the agar plates as cleared areas and in the membrane *strips* as *dissolved* haemoglobin

bands, which were readily adsorbed. The quantitative assay was made by performing the above procedure under standard condition at 37° for 60 min. The haemoglobin fraction was cut out and eluted from the membrane fragments with 0.5 ml of 0.01 N-NaOH. The eluate was neutralised with 0.5 ml of 0.1 N-H_2SO_4 and the haemoglobin estimated by the iron method.

The results were expressed in terms of mg of haemoglobin liberated by the venom fraction, under standard conditions.

Results and Discussion

The electrophoretic patterns of *Vespa orientalis* venom sac extract venom and *Paravespula germanica* venom are shown in fig. 1 while the quantitative results appear in table 1. A maximum number of eleven different fractions was found in the samples examined. We therefore divided the pherogram – by parallel lines – into eleven zones, starting with the one nearest to the anode and numbered accordingly. Fractions of the different specimens falling into the same zone may be identical both in structure and in biological activity: in many cases, however, they are probably different. For this reason they are considered as identical, or similar, only with regard to electrophoretic mobility.

The differences in the electrophoretic patterns are obvious at a glance. The main protein fractions in *Vespa orientalis* venom are basic proteins migrating in the direction of the cathode, and they represent more than 70 % of the total proteins. In *Paravespula germanica* venom, though signif

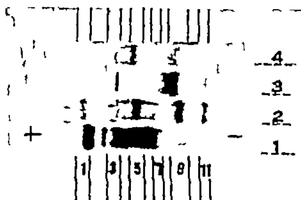


Fig. 1. Microzone electrophoresis of wasp venom. Vertical numbers: 1. Human serum. 2. *Vespa orientalis* venom sac extract. 3. *Vespa orientalis* venom. 4. *Paravespula germanica* venom. Horizontal numbers: Represent the zonal division of the eleven fractions detected in the samples. The sharp line which is seen in the venom samples at zone 3 is an artefact caused by denaturated proteins and has therefore been discarded.

Table 1

The protein content of wasp venom fractions separated by electrophoresis. The relative (%) and absolute (mg%) protein content of proteins found in the *different fractions* by the membrane electrophoretic separation method described in detail in the text.

Zone division ⊥		1	2	3	4	5	6		8	9	10	11
Paravespula germanica venom	mg%		36.0		42.0	293.0		8.0		110.0		11.0
	%		7.3		8.5	58.3		1.6		22.0		2.3
Vespa orientalis venom	mg%			38.0		26.0		22.0	69.0	263.0	28.0	54.0
	%			7.6		5.2		4.4	13.8	52.6	5.7	10.7
Vespa orientalis venom sac extract	mg%	55.0		11.0	31.0	158.0	17.0	17.0	27.0	126.0	20.0	57.0
	%	10.5		2.2	6.3	31.7	3.4	5.3		25.2	4.1	11.3

Note: Each sample contains 500 mg% of total protein.

icant amounts of basic proteins are present, the fractions migrating towards the anode are in excess of these. It is, moreover noteworthy that the dye uptake of this venom was lower than in other samples and, though the total protein concentrations were the same, the fractions were more faintly stained.

In *Vespa orientalis* sac extract one would expect to find the same fractions as in the pure venom, along with the addition of a number of proteins derived from the tissues. Yet thus is not exactly the case. Thus fractions 11 and 10 are similar but the major fraction, no. 9 moves closer towards the cathode, suggesting a gain of positive radicals. Fraction 8 a significant component in pure venom, is absent from the extract, while fraction 5 is a major component despite the fact that it is present in traces only in the pure venom. A considerable amount of an albumin-like substance is found in the extract however since it moves a little faster it is more likely to be a prealbumin.

These results suggest that extraction of the venom from whole sacs not only introduces foreign proteins, but that treatment causes denaturation or modification of some of the components, and removes some fraction, possibly by adsorption to the stroma or to some of the denaturated proteins. Since a possible alteration or loss of some biological characteristics of the venom may occur we are of the opinion that all the biological data

obtained by using venom sac extract (SHULMAN *et al.* 1964) should be evaluated in the light of these results and that, in further studies, only pure venom should be used, until convincing evidence is available that our assumption is invalid.

In *Vespa orientalis* venom three major protein fractions possess haemolytic activity (fig. 2) All three fractions are basic proteins migrating towards the cathode. Their position coincides with the zone numbers designated as 8 9 and 10 We shall refer to them as haemolytic fractions B, C and D i.e. in the same order as their anode-to-cathode position. The highest protein content, as well as most of the haemolytic activity was found in fraction C, the haemoglobin protein ratio – meaning the haemolytic activity per unit of protein – however being lowest in this component (table 2) The haemoglobin protein ratio was the highest in the most basic D fraction, the least basic B having an in-between position in this respect.

Examination of the venom sac extract showed the presence of fractions C and D but in the position of B no haemolytic activity was detected. Instead of a new haemolytic fraction, A appeared in the protein zone 5 This phenomenon suggests that venom proteins undergo an alteration during the extraction process. Since both the concentration of proteins

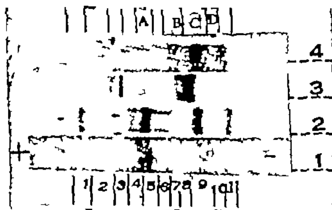


Fig. 2. The relation of the haemolytic factor to protein fractions of the wasp venom. *Vertical numbers:* 1. Haemolytic components in *Vespa orientalis* venom sac extract. 2. The protein fractions of same. 3. *Vespa orientalis* pure venom fractions. 4 The haemolytic factors associated with the pure venom proteins (of no. 3).
Horizontal numbers: Zonal division of the separated proteins A, B, C and D haemolytic fractions roughly corresponding to the fractions 5, 8, 9 and 10 of the protein zone. Note the shift of the chief fraction in the venom sac extract. Minor haemolytic fractions seen in the picture have not been discussed in the paper, since a *quantitative* estimation was not feasible.

Table 2

The haemolytic activity of *Vespa orientalis* venom and venom sac extract protein fractions.

Vespa orientalis		Fraction A	B	C	D
Venom	Total protein mg%	26.0	69.0	263.0	54.00
	*Haemoglobin** mg	–	235.0	410.0	225.00
	Hb/Prot.	–	3.4	1.6	4.15
Venom sac extract	Total protein mg%	158.0	–	126.0	57.00
	Haemoglobin mg	415.0	–	204.0	230.00
	Hb/Prot.	2.6	–	1.6	4.05

mg of haemoglobin liberated under standard condition of 60 min. at 37° by 1 µl of sample containing 500 mg% of total protein.

and the amount of haemoglobin liberated by the active principle are higher in this new fraction than they were in fraction B of the pure venom, the balance obviously has to come from fraction C.

From the above data it is concluded that the haemolytic activity is not proportional to the increasing basicity of the protein containing it and that though the loss of positive radicals due to denaturation changes the electrophoretic mobility the haemolytic principle is not significantly altered.

The question whether the haemolytic principles are proteins or are attached to various protein fractions however remains unanswered. The complete transformation of fraction B of the pure venom into fraction A of the sac extract, and the fact that on dialysis, no haemolytic activity was detectable in the dialysate, support the former view However the partial denaturation of fraction C and the excessive activity and proteins in the same (A) fraction obscures the overall picture. In either case, it is probable that the antibodies effective against the haemolytic factor could be produced in experimental animals. Such a project is being undertaken by our group and the results will be reported in a subsequent paper.

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From the Department of Physiology and Pharmacology, Medical School, Tel-Aviv University, Tel-Aviv and the Department of Biochemistry, Beilinson Hospital, P.O.B. 85, Petach-Tikva, Israel

Investigation of Wasp Venom Antigenic Relationship

By

J. Ishay*, J. Fischl and S. Gitter

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Abstract. Rabbits immunised with venom of *Vespa orientalis* developed antibodies related to four of the protein fractions of the venom and gave well-defined precipitin reaction in immuno-electrophoresis and immuno-diffusion. Three of the immuno-proteins present were contained in the same electrophoretic fractions which containing the haemolytic principle of the venom. The fourth was identified as lysozyme. *Paravespula germanica* venom reacted with the antiserum showing two precipitation bands, indicating partial inter species cross-immunity.

Key-words: Wasp venom – antigens – immunoelectrophoresis – immunodiffusion.

Wasp venom is a complex biological substance producing many physiological effects when injected into experimental animals (Edery & Ishay 1965). In addition to being lethal to most insects as well as to some domestic animals, human subjects are also vulnerable, and death due to nephrotoxic, hepatotoxic and allergic involvement may occur (Jonas & Shugar 1964; Schaller 1964).

The predominant and most offensive wasp species in the Middle East is the Oriental Hornet *Vespa orientalis* (Ishay 1964). The immunological properties of related species have been investigated by other workers (Langlois *et al.* 1965).

In this paper we present the immunological reactions obtained in rabbits with the *Vespa orientalis* venom, the cross-reaction of the antiserum with *Paravespula germanica* venom, and the relationship of the immunoproteins with the haemolytic factor.

Address: Department of Physiology and Pharmacology, Beilinson Hospital, P.O.B. No. 85, Petach-Tikva, Israel.

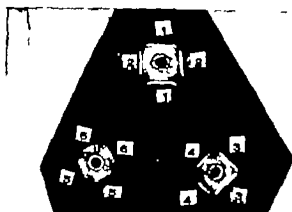


Fig. 1. Response of six (no. 1–6) rabbits to immunisation with *Vespa orientalis* venom. Central well: *Vespa orientalis* venom. Outer wells: Rabbit *Vespa orientalis* venom antiserum. Note the identity of duplicates and the slight individual differences.

Methods and materials

Methods of venom collection, electrophoresis and the determination of the haemolytic activity have been described in previous papers (FISCHL *et al.* 1972).

Antiserum was obtained by immunizing six rabbits with *Vespa orientalis* venom for five weeks, with a dose of 10 μg of venom with Frend's adjuvant twice weekly and a final booster dose of 100 μg as the last injection. After immunization the animals were bled, the serum separated by aseptic techniques, merthiolate added (1 10,000) and the serum then stored in a refrigerator.

Immuno-diffusion was performed on Ouchterlony's plates, using 1.2% "Difco Noble Agar in 0.05 M barbital (diemalum NFN) buffer (pH 8.6). The diameter of the outer wells was R mm and of the center one of 3 mm, the distance between wells was five mm and the agar's thickness R mm. The same agar solution was also used in immuno-electrophoresis.

Lysozyme was prepared from human urine and rabbit antilysozyme serum was produced from the above.

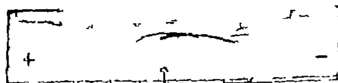


Fig. 2. Immuno-electrophoresis of *Vespa orientalis* venom. The trough contains pooled rabbit, antivenom serum. The venom was placed into the well (indicated by the arrow). Note the presence of at least four (possibly five) precipitation bands.

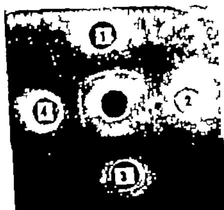


Fig. 3 Identification of lysozyme in wasp venom.
Central well. rabbit-antilysozyme serum. Wells 1 and 4 lysozyme.
Wells 2 and 3. *Vespa orientalis* venom.

Results and discussion

All six rabbits responded similarly to the immunisation, though quantitative differences were found (fig. 1). On immunoelectrophoresis, four precipitation arcs were observed (fig. 2) all of them in the direction of the cathode. The position of the immuno-fractions corresponded to the protein zones 8, 9 10 and 11 of the division described in our previous paper (FISCHL *et al* 1972) and with fractions B, C and D of the haemolyzing fractions (FISCHL *et al.* 1972).

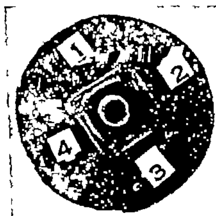


Fig. 4. Indication of cross-immunity of *Vespa orientalis* and *Paravespula germanica* venoms. Central well. *Vespa orientalis* antivenom serum (rabbit). Wells 1 and 3 *Vespa orientalis* venom. Wells 2 and 4 *Paravespula germanica* venom. Note the identical lines of at least two immuno-fractions.

Fraction 11 which is devoid of haemolytic activity has an electrophoretic mobility identical with, or close to lysozyme. The identity and thus the presence, of lysozyme in wasp venom was supported by evidence obtained by the immunodiffusion method (fig. 3)

Paravespula germanica venom reacted with the antiserum, giving two precipitin arcs, which suggests partial cross-immunity (fig. 4)

It is realised that wasp venom contains a great number of toxic substances of diverse physiological activity Many of these are not proteins. Hence it cannot be anticipated that any antiserum will give complete protection against all the toxic effects. Nevertheless, the powerful haemolytic property is undoubtedly an important factor in toxicity and might be counteracted by the antiserum.

Further experiments are currently in progress.

Acknowledgement

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From the Department of Neurology, Bispebjerg Hospital, DK-2400 Copenhagen NV
Denmark

Effects of Ergot Alkaloids (Hydergine®) on Cerebral Haemodynamics in Man

By

Jes Olesen and Erik Skinhøj

(Received July 23, 1971; Accepted October 20, 1971)

Abstract. The intracarotid ^{133}Xe injection method gives a very accurate determination of regional blood flow within the brain and can be used in human subjects when awake. By this method it has been demonstrated that the ergot alkaloid preparation hydergine® has no immediate effect on blood flow through normal cerebral tissue and no direct effect on diseased areas. However secondary to an ergot initiated decrease in arterial blood pressure, cerebral blood flow is decreased in areas with impaired auto-regulation. The difference in the blood flow response between diseased and non-diseased areas illustrates the need for regional measurements in studies of the pharmacology of the cerebral circulation.

Key-words: Ergot alkaloids – hydergine® – cerebrum – haemodynamics.

Hydergine® is composed of equal parts of the three hydrogenated ergot alkaloids i.e. dihydroergocornine, dihydroergocristine, and dihydroergocryptine, and it is thus in principle an adrenergic blocking agent. In some clinical trials hydergine® has been claimed to be of benefit in senile cerebral vascular disorders. This has been attributed to the cerebral vasodilator effect of the drug. The concept of ergot alkaloids as vasoactive agents which act on the cerebral vessels is, however based on rather poor and controversial experiments. The purpose of the present study was to evaluate the effect of hydergine® on the haemodynamics in normal and diseased brain tissue, and at the same time to introduce the intracarotid ^{133}Xe injection method for the determination of regional cerebral blood flow as a tool in pharmacological studies.

Material and methods

14 patients in whom a carotid arteriography was indicated were examined. The regional cerebral blood flow (rCBF) measurements were done in combination with

angiogram. In 9 of the cases we used an intravenous administration of hydergine® in 4 an intracarotid infusion and in one patient (case 5 in table 1) both intravenous and intracarotid administration. The diagnoses were: Cerebral apoplexy migraine, dementia due to an organic, but not vascular lesion and one tumor case

The regional cerebral blood flow (rCBF) was measured by the intracarotid ^{133}Xe injection method (LASSEN & INGVAR 1961 LASSEN *et al.* 1963, HOEDT-RASMUSSEN *et al.* 1967 PAULSON 1970 OLESEN & PAULSON 1971 OLESEN *et al.* 1971). No premedication was given. Under local anaesthesia (lidocain 1%) the common carotid artery was punctured and by means of the Seldinger technique a thin polyethylene catheter was placed into the internal carotid artery The correct position was checked by a rapid injection of saline which causes a blanching in the supraorbital region when injected into the internal carotid. A rapid injection of 2-3 mci ^{133}Xe dissolved in 2-5 ml of isotonic saline was given and the wash out of radioactivity from the hemisphere was followed by 35 small scintillation detectors placed laterally over the ipsilateral side of the head. The detectors are collimated with cylindrical lead tubes 43 mm long and 12 mm in internal diameter and consist of NaI crystals 12 mm in diameter and 10 mm thick. The counting field of one detector is then represented by a narrow truncated cone transsecting the entire hemisphere. At a distance of 3 cm from the collimator approximately corresponding to the surface of the brain – the diameter of the cone is 2 cm. 65% of the counts are taken from this compartment whereas 35% consists of compton scatter This tends to smooth out any regional differences in blood flow Because of absorbtion of irradiation in the tissues the superficial structures (cortex) are moderately over represented in the clearance curves. The detectors are packed together in a rectangular box (fig. 1) of a size which can cover a large human head. Thus, usually a few channels were too peripheral and had to be discarded because of low counting rate. The position of the detectors was marked on the head of the patient and after the study small pieces af lead were taped on the head, thus marking the position of the detectors on a lateral projection x-ray film.

The impulses from all 35 detectors are displayed logarithmically via a multiplexer on an oscilloscope screen and photographed with a polaroid camera. The results are thus immediately available as a photographic picture of 35 clearance curves, each representing by their slope the blood flow through a small part of the brain (fig. 1).

The sum of the output of all channels is displayed on a potentiometer writer which runs continuously during the measurement and from which the degree of remaining activity from previous isotope injections can be assessed.

Usually 4 measurements were performed in each of the individual patients at 15 minutes interval This time is required in order to bring down the radioactivity from the preceding isotope injection. The order of the measurements was usually rest-test-rest-test.

In one group of patients intravenous injections of 1-3 ml were given at varying times before the flow measurement. In another group, the drug was given as a continuous infusion into the internal carotid artery Doses and infusion times are listed in tables 1 and 2.

After the flow study carotid angiography was finally performed through the indwelling catheter

Calculations.

If the clearance of ^{133}Xe is followed for 10 to 15 minutes the cerebral blood flow can be calculated according to the stochastic analysis (height-over-area) or to the bicompartmental analysis (proportional to the slope of the monoexponentials represent-

ing gray and white matter respectively (fig. 2). A more rapid and yet very accurate method for the estimation of the cerebral blood flow is, however to measure the slope of the first 1–2 minutes of the logarithmically displayed clearance curve ($rCBF_{initial}$). The flow values obtained by this method are closely correlated to the values obtained by the two other calculations (OLESEN *et al.* 1971), and because of the short recording time a steady state of respiration (P_{aCO_2}), *blood pressure and drug effects can more easily be ensured.*

The principle underlying the initial slope calculation is that *the first 1–2 minutes of the clearance curve from the brain can with great accuracy be regarded as monoexponential since the gray matter is dominant. Thus the flow can be calculated as done when dealing with homogeneous tissue:*

$F = a\ \lambda\ 100$ (ml/100 g/min.) where a is the slope of the logarithmically displayed curve (natural logarithm) and λ the tissue to blood participation coefficient.

Applying this equation to the cerebral clearance curves and using base 10 logarithm:

$CBF_{initial} = 2.3\ D_{initial}\ \lambda_g\ 100$ (ml/100 g/min.) where 2.3 is the conversion factor from base 10 to natural logarithm and $D_{initial}$ the slope of the first 1–2 minutes of the clearance curve and λ_g the partitition coefficient of gray matter. With a fix $\lambda_g = 0.87$ and calculating the slope in per cent of a decade per minute the equation is reduced to:

$CBF_{initial} = 2\ D_{initial}$ *ml/100 g/min.*

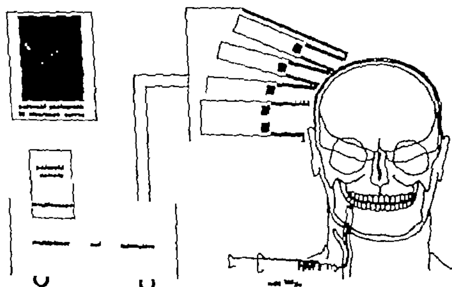


Fig. 1. Schematic drawing of the ^{133}Xe intra-arterial injection *method for regional cerebral blood flow* (rCBF) measurements. A bolus of ^{133}Xe dissolved *in saline is injected rapidly into the internal carotid artery* and the washout of radioactivity *followed by multiple collimated scintillation* detectors. A curve from each small area of the brain is displayed *logarithmically* on an oscilloscope screen and photographed by a polaroid camera. The $rCBF_{initial}$ is proportional to the slope of these curves.

Table 1

Effect of hydergine® on the regional cerebral blood flow Intravenous injection.

			MABP (mmHg)		$PaCO_2$ (mmHg)		Mean $rCBF_{global}$ (ml/100g/min.)		
Case no.	Diagnosis	Dose (ml)	rest	test	rest	test	rest	test	test corrected for $PACO_2$
1	Migraine	3	73	75	40.5	40.4	71	67	67
	Migraine	.	90	78	40.3	41.5	54	53	51
3	Apoplexy	2	76	58	41.4	45.0	55	58	50
4	Apoplexy	3	124	122	38.2	41.7	38	40	34
5	Apoplexy	2	94	100	40.2	39.5	58	57	59
6	Apoplexy	2	105	90	36.4	37.4	40	33	32
7	Apoplexy	1	95	80	37.7	41.3	66	68	58
8	Apoplexy	2	135	125	40.2	45.2	36	35	28
9*	Migraine	2	173	150	31.0	29.0	110	103	111
10	Cerebral metastasis	2	70	58	38.4	40.0	23	25	24
Mean			104	94	38.4	40.1	55.1	53.9 NS	51.4 P<0.05

Cases with focal abnormalities.

Table 2

Effect of hydergine® on the regional cerebral blood flow Intracarotid infusion.

			MABP (mmHg)		$PaCO_2$ (mmHg)		Mean $rCBF_{global}$ (ml/100g/min.)		
Case no.	Diagnosis	Dose (ml)	rest	test	rest	test	rest	test	test corrected for $PaCO_2$
1	Apoplexy	0.05	102	95	48.3	47.2	64	64	67
2	Apoplexy	0.10	94	86	40.2	38.4	58	52	56
3	Dementia	0.025	75	75	39.7	36.5	60	74	83
4	Dementia	0.10	81	78	41.5	41.7	71	66	64
5	Apoplexy	0.10	88	82	43.2	46.0	60	63	56
Mean			88	83	42.6	42.0	62.6	63.8 NS	65.4 NS

Thus the cerebral blood flow is proportional to the slope of the logarithmically display clearance curve and can be read directly from the films in a simple apparatus consisting of a disc which is moved in such a manner that a line fits the curve.

When two flow results are to be compared it is necessary to correct for possible changes in arterial pCO_2, since this factor is of utmost importance in the regulation of the cerebral circulation. *The correction was performed as a 4% flow change per* mmHg change of $PaCO_2$ (Olesen *et al.* 1971). Correction for the remaining radioactivity from previous isotope injections was also carried out (Olesen et al. 1971).

The reproducibility of flow values with the present technique applied to patients with various diseases has been estimated by repeated resting state flow measurements in a group of patients. *Between the two resting state measurements a pharmacological* test was carried out so that the experimental conditions were comparable to the present study. In a single channel the flow value was reproduced with a change of ± 7.6% and for the average hemispheric flow value the reproducibility was ± 5.1% (Olesen *et al.* 1971). These figures are used in the statistical calculations.

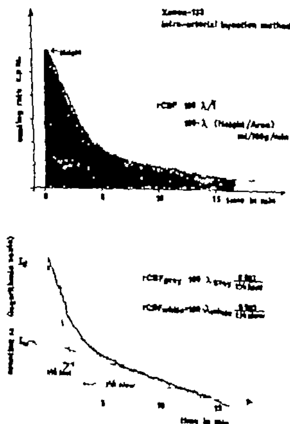
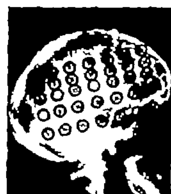


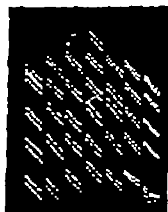
Fig. 2. This figure *demonstrates the two classical* ways of calculating the cerebral blood flow from a ^{133}Xe clearance curve from the brain. The upper curve is displayed *in the linear mode* and shows the height-over-area method. The lower curve is displayed logarithmically and shows the bi-compartmental analysis with calculation of flow in gray and white matter, separately.

Results

The range of cerebral blood flow during the resting state varied from extremely low values (cases 4 8 10 in table 1) through normal values to an extreme case of "luxury perfusion" (HOEDT RASMUSSEN *et al.* 1967) (case 9) The depression of global flow values and metabolism in cases with only focal disturbances is in agreement with our previous studies (HOEDT RASMUSSEN & SKINHØJ 1964) Three of the patients showed focal abnormalities i.e. an area where the perfusion differs significantly from the remainder of the brain at rest and/or during the functional tests mentioned below

EFFECT OF HYDERGINE ON CEREBRAL BLOOD FLOW





	REST	HYDERGINE
MABP (mm Hg)	75	58
$PaCO_2$ (mm Hg)	43.1	45.0
$CBF_{initial}$ (ml/100g/min)	59	59

Fig. 3. The position of the 35 detectors over the head is seen to the left (compare site with the anterior view in fig. 1). To the right, two subsequent flow determinations are superimposed. Each pair of curves represents the flow in one small part of the brain. The upper *curves in each pair are measured* in the resting state and the lower curves *following* the injection of hydergine® The slopes of the curves are practically identical and the calculated blood flow is the same. The patient is not included in the tables.

Table 3

Effect of hydergine® on the regional cerebral blood flow in patients with focal vasoparalysis.

Case no.	Diagnosis	Number of focal channels	Flow change in focus %	Flow change outside* focus %
8	Apoplexy	4	−25	−8
9	Migraine	10	−19	0
10	Cerebral metastasis	2	−59	0

No correction for remaining radioactivity or $PaCO_2$ change has been carried out here.

It appears from table 1 that hydergine® is a rather potent vasoactive drug which in therapeutic doses reduces the mean blood pressure by approximately 10 per cent. At the same time the cerebral blood flow decreases only insignificantly (fig. 3). When correction for a concomitant increase in $PaCO_2$ is carried out the decrease is longer and significant ($P < 0.05$ paired t-test)

The explanation for the marked decrease of CBF in patients 6 and 7 undoubtedly was a globally impaired autoregulation – as seen in most acute cases of severe apoplexy With an abolished autoregulation the reduction in blood pressure induced by hydergine® results in an equal reduction in cerebral blood flow In case 8 the fall in the mean hemispheric blood flow was mainly due to a focal loss of autoregulation and a focal decrease in blood flow (table 3).

The intracarotid administration of hydergine® (table 2) was undertaken so that the drug could be used in very small doses. This would provide high local concentrations in the cerebral vessels but minimize any systemic effects. *Even with small doses a decrease in the mean arterial blood pressure* was, however observed. The intra-carotid infusion of hydergine® did not cause a major change in CBF in any of the patients except in case 3 where an increase of 38% was found. This patient who was relaxed during the resting state study became agitated and hyper-ventilated during the pharmacological test, which might account for the increase in CBF (after correction for pCO_2 change). In rare cases in other series we have seen this increase in CBF associated with agitation and tentatively consider it as being due to a central "arousal" rather than to a specific reaction to the drug itself For the group as a whole no significant change in CBF occurred whether correction for $PaCO_2$ was carried out or not.

The results obtained in patients with focally abnormal resting state rCBF patterns are listed in table 3 It appears that the flow in focal areas

creased due to hydergine® whereas it stays constant in the non-affected parts of the hemisphere. The decrease in flow must therefore be attributed to focal loss of autoregulation in combination with the hypotensive effect of hydergine® rather than to a constrictor effect on the cerebral vessels.

Such differences between regions within the same cerebral hemisphere illustrate why determinations of cerebral blood flow from multiple small areas of the brain are necessary for proper evaluation of the effect of drugs on the cerebral circulation. Merely to measure the average hemispheric blood flow may be quite misleading.

Discussion

In some clinical studies (ABADAS 1963 DITCH *et al.* 1971 GERIN 1969 GRILL & BROICHER 1969 HEYCK 1962 HOFFMEISTER 1967 SCHMIDL & OSWALD 1967 SANDER TRESKE 1963 TRIBOLETTI & FERRI 1969) hydergine ® is claimed to have a beneficial effect in patients with symptoms resulting from cerebral vascular insufficiency. A critical review of these publications, however does not leave a very convincing impression. The studies either do not include a control group or it is not clear whether the treated and non-treated groups are equally matched. The latter point is crucial, since the treated patients showed complex clinical pictures with varying degrees of somatic and psychological problems.

During recent years the medical research division of "Sandoz" has initiated a study in eleven centres in the USA and Europe on the clinical effects of hydergine® in cerebrovascular insufficiency (ULRICH 1971 personal communication). 41 clinical parameters were evaluated. The pooled data indicated that in 36 of these parameters, hydergine® gave a more favourable response than placebo. However only 21 of the parameters were significantly improved ($P < 0.05$) including symptoms such as confusion, mental alertness, periods of depression mood, motivation and initiative, hostility, indifference to surroundings, lack of sociability lack of cooperation, anxiety and fear anorexia, dizziness and vertigo, incoordination of movements, difficulty in walking, difficulty in dressing, self care, and overall impression of the patients.

In almost all the above mentioned clinical studies the authors take it for granted that hydergine® has a dilator effect on cerebral vasculature resulting in an increase in cerebral blood flow. From a theoretical point of view however an effect of hydergine® on the cerebral blood flow would not be expected. In spite of the fact that the sympathetic innervation of the cerebral arteries has been well demonstrated there is no evidence that these nerves act as vasoconstrictors under normal circumstances. Thus YORDANOW &

VLAHOV (1971) found no change in the cerebral blood volume of the cat after blockade with phentolamine. In man an alpha adrenergic blockade with phentolamine does not cause any change in the cerebral blood flow in autoregulation or in the CO_2 response (SKINHØJ 1971 unpublished results). In patients with destroyed sympathetic function – advanced cases of Shy-Drager syndrome – the cerebral autoregulation is unaffected (SKINHØJ *et al.* 1971). Finally a normal cerebral blood flow autoregulation and pCO_2 responses are present in animals following chronic sympathetic denervation (WALTZ *et al.* 1971 EKLÖF *et al.* 1971).

The effect of hydergine® on cerebral blood flow in man has been investigated by HEYCK 1961 & 1962), who used the Kety-Schmidt method. He found an increase of 13 per cent after intravenous administration of hydergine® most pronounced in cases with reduced flow values. GERAUD *et al* (1963) used a modification of the Kety method and confirmed the results of HEYCK. In neither of these two reports are any data given concerning the arterial pCO_2 values during the study. This is *critical* information as an increase of only one mmHg in arterial pCO_2 gives an increase around 4 per cent in cerebral blood flow (OLESEN *et al.* 1971) GOTTSTEIN, in his careful and extensive study "Der Hirnkreislauf unter dem Einfluss vasoactiver Substanzen" (GOTTSTEIN 1962) did not find any effect of hydergine® in man either by intravenous or by intracarotid administration. These three investigators all used about the same dose. EMMENEGGER & MEIER RUGE (1968) in the cat found an increase in cerebral blood flow around 10 per cent after hydergine® but his experimental conditions were rather unphysiological as the cats were kept very hypothermic. SZEWCZYKOWSKI *et al.* (1970) in baboons and using a flowmeter technique found an increase in blood flow of approximately 8 per cent, but the changes were only of 10 minutes duration and they were only found when using doses which were much higher than those used clinically

As seen in the present studies hydergine® does not *affect the* cerebral blood flow when corrected for possible changes in arterial pCO_2 if flow is low normal or high, either in the normal brain or in the normal parts of a diseased brain. In the diseased parts of the brain with a lost autoregulation it has no specific haemodynamic effect, but it decreases blood flow secondary to a decrease in blood pressure.

The clinical effect in patients – if any – must therefore be ascribed to other properties of the drug than its haemodynamic effects.

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From the Department of Pharmacology (Professor C. G. Schmiterlöw M. D.), Royal Veterinary College, S-104 05 Stockholm 50, Sweden

The Distribution of Labelled Aldosterone in Mice Using Whole Body Autoradiography

By

Lars-Erik Appelgren and Evangelina Marrero

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Abstract. Whole body autoradiography of mice 2, 20 and 60 minutes after intravenous injection of ^{14}C-4-aldosterone showed a rapid and high accumulation of aldosterone and/or its metabolites in the liver bile and kidney indicating the routes of excretion. Besides the uptake in the kidney and intestine, well known as target organs for aldosterone, a high uptake was also registered in ductus deferens, caput epididymidis and adrenal cortex. Thin-layer chromatography of chloroform extract of selected tissues showed high amounts of unchanged aldosterone 2 minutes after the injection, but after 20 minutes scintillation counting showed that most of the radioactivity was found in the water phase and/or bound to the tissue residues.

Key-words ^{14}C-aldosterone – distribution – autoradiography

In previous distribution studies with labelled aldosterone, the uptake of radioactivity was found chiefly in the kidney liver and intestine indicating routes of excretion (SULYA *et al.* 1963 MCCAA *et al.* 1964)

Micro-autoradiographic experiments with toad bladder incubated in ^{3}H aldosterone have shown a localization of radioactivity in the nuclei of the epithelium (EDELMAN *et al* 1963)

Since the weaker mineralocorticoids cortisol and cortisone have been shown to have an affinity to some tissues not generally regarded as target sites, e.g. the epididymis and adrenal cortex (HANNGREN *et al.* 1964) it was also thought of interest to study the whole body distribution of aldosterone.

A whole body autoradiography study was therefore made and in this connection some chromatographic and scintillation counting experiments were made on chloroform extracts of some selected tissues.

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* From C. N. I. C., Habana, Cuba, holding a SIDA-fellowship.

Materials and Methods

The labelled compounds used were ^{14}C-4-aldosterone, specific activity 56.7 mci/mM obtained from the Radiochemical Centre, Amersham, England, and ^{3}H 1,2-aldosterone, specific activity 50 ci/mM obtained from New England Nuclear Boston, Mass., USA.

Autoradiography

Three albino mice (NMRI) were injected intravenously with 2 μci of ^{14}C-4-aldosterone dissolved in 25 μl ethanol according to the method described previously (APPELGREN 1967). The survival times were 2, 20 and 60 minutes. The animals were anaesthetized with chloroform and sacrificed by immersion in hexane cooled to −78 with solid CO_2. The mice were embedded in a mixture of carboxymethyl cellulose and water before freezing. Sagittal sections (20 μ and 60 μ) of the whole body were cut at −15° according to the Ullberg autoradiographic technique by which each section is attached onto tape (No. 810 Minnesota Mining and Manufacturing Co.) (ULLBERG 1954 & 1958). The sections were then dried at −15° and pressed against x-ray films (Structurix, Gevaert and Crystallex, Kodak) and stored in a press at the same temperature.

In order to void the artefacts sometimes seen in autoradiograms, due to the melting of the fat at room temperature when a fat soluble substance is used, all the dark room work, except for developing, was carried out at −15°

Another male mouse was injected with 2 μci of ^{14}C-4-aldosterone and the testicle, epididymis and ductus deferens from both sides were removed after a survival time of 20 minutes. The organs were immediately frozen and embedded in carboxymethyl cellulose, sectioned and autoradiographed following the technique described above for whole body autoradiography. In addition some sections of this specimen were attached to Ilford G 5 plates. The exposure time varied from 14 days to 3 months. ^{3}H-1,2-aldosterone (0.5 mci) was injected intravenously into 1 male mouse using the same technique as that used for ^{14}C-aldosterone. After a survival time of 2 minutes the mouse was prepared for whole body autoradiography. Ten μ sections were cut and attached onto tape (No. 688, 3 M Company) and dry mounted onto G 5 plates (Ilford) (cf. HAMMARSTRÖM *et al.* 1965 APPELGREN 1967). Before developing, the tape was separated from the photographic plate by immersion in xylene, leaving the section in contact with the emulsion.

Quantitation and chromatography

Three male albino mice (NMRI) were injected intravenously with 2 μci ^{14}C-4-aldosterone dissolved in 25 μl ethanol as described above. One animal was sacrificed after 2 minutes and the remaining 2 animals after 20 minutes, by stretching their spines. The following tissues were collected. epididymis, adrenal, intestine, testicle, kidney blood, salivary gland, liver bile and ductus deferens. The samples were immediately weighed and transferred to a freezer (−20°) after the addition of 1 ml 0.9% NaCl solution. The samples were then homogenized in a Potter-Elvehjem homogenizer and extracted twice in chloroform. The recovery of the ^{14}C-4-aldosterone added to the homogenized tissue was found to be about 70% in the chloroform phase.

After centrifugation, the chloroform layer was sucked off with disposable pipettes and evaporated to dryness with N_2. The residue was re-dissolved in 1 ml chloroform and 25 μl was counted in a liquid scintillation spectrometer (Tricarb 3003 Packard) after the addition of 10 ml of 0.5% PPO in toluene. Samples obtained from the water phases were also counted. The radioactivity in the remaining tissue rests was checked after rinsing with 0.9% saline solution. The tissue was digested with perchloric acid and counted according to the method of MAHIN & LOFBERG (1966).

The chloroform extracts were chromatographed on thin layer (200 µ) plates of silica gel G using chloroform-acetic acid (90:10) or chloroform-aceton-acetic acid (70:20:10) as developing agents. The radioactivity of the chromatograms was detected by autoradiography and/or the aid of a radiochromatogram scanner (Packard 7201).

Non-radioactive d-aldosterone was used as a reference substance. Since variations in the Rf-values were seen, the reference substance was run in the same plate as the radioactive tissue extracts in order to facilitate comparison. The chromatograms of the non-radioactive substance were made visible by spraying the plates with sulphuric acid-methanol (1.1). After treating the plates for 20 minutes at +100 they were studied in daylight. In some cases the relative concentrations of the different spots were determined by scraping out the spots localized by scanning and counting them by liquid scintillation according to APPELGREN (1967)

Results

Autoradiography

The radioactivity disappeared from the blood very rapidly and as early as 2 minutes after the injection the concentration was about half that of the myocardium. The highest uptake of aldosterone and/or its metabolites was seen in the bile and ductus deferens. Moreover a high concentration was also registered in the liver intestinal contents, caput epididymidis, adrenal cortex, Meibomial glands and bronchi. Lower amounts of radioactivity were found in the pancreas, salivary glands, teeth, pituitary gland, myocardium, choroid plexus and interstitial cells of the testicle.

After 60 minutes the radioactivity was mainly found in the liver and intestinal contents thus indicating the routes of excretion. At the same time a low concentration was observed in the kidney The ductus deferens, however showed a rather high concentration of ^{14}C (fig. 4) The distribution patterns of the two differently labelled compounds were in good agreement.

The distribution in various tissues at different time intervals is given below

Skeleton. Bone and *cartilage* showed a very low concentration of ^{14}C at all the times studied. In the *bone marrow* the radioactivity slightly exceeded that of the blood.

Muscles The *skeletal muscles* had about the same concentration as the blood after 2 and 20 minutes. After 60 minutes no radioactivity was detectable. The *myocardium* had a higher uptake than the blood during the whole experiment.

Respiratory organs The *bronchi* had a concentration of radioactivity which was higher than that of the myocardium, but less than that of the liver 2 and 20 minutes after injection. The *parenchyma of the lung* had the same concentration as that of the blood.

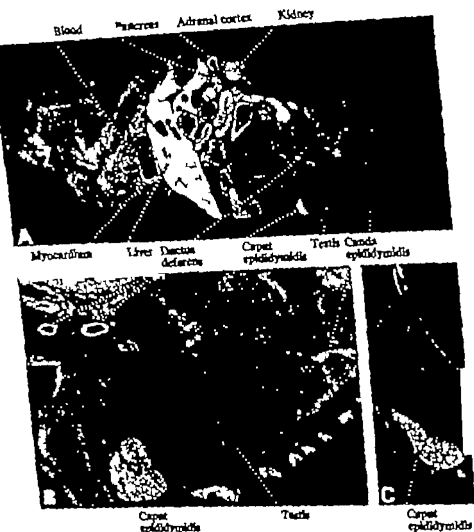


Fig. 1A. Autoradiogram of a male mouse 2 minutes after injection of ^{14}C-4-aldosterone. Note high concentration of radioactivity (white areas) in the liver adrenal cortex and ductus deferens.

Fig. 1B Detail of fig. 1A, showing the high uptake in the ductus deferens, parts of the caput epididymidis and the interstitial cells of the testis.

Fig. 1C. Detail of a whole body autoradiogram of a mouse 2 minutes after intravenous injection of ^{3}H-1,2-aldosterone showing the uptake in the caput epididymidis, mainly confined to the epithelium.

Digestive system. The *teeth* had the same concentration of ^{14}C as the blood. The *salivary glands* showed an accumulation similar to that found in the myocardium 2 and 20 minutes after the injection of labelled aldosterone. The *liver* showed marked accumulation of labelled aldosterone throughout

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Results

Autoradiography

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After 60 minutes the radioactivity was mainly found in the liver and intestinal contents thus indicating the routes of excretion. At the same time a low concentration was observed in the kidney. The ductus deferens, however, showed a rather high concentration of ^{14}C (fig. 4). The distribution patterns of the two differently labelled compounds were in good agreement.

The distribution in various tissues at different time intervals is given below.

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Muscles. The *skeletal muscles* had about the same concentration as the blood after 2 and 20 minutes. After 60 minutes no radioactivity was detectable. The *myocardium* had a higher uptake than the blood during the whole experiment.

Respiratory organs. The *bronchi* had a concentration of radioactivity which was higher than that of the myocardium, but less than that of the liver 2 and 20 minutes after injection. The *parenchyma of the lung* had the same concentration as that of the blood.

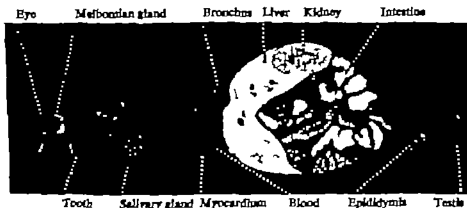


Fig. 2. Autoradiogram of a male mouse 20 minutes after intravenous injection of ¹⁴C-4-aldosterone. Note the uptake in the liver, bile, kidney and intestinal contents.

Quantitation and chromatography

The results of the liquid scintillation of the combined chloroform and water extracts are shown in fig. 5. The relative concentration of radioactivity in the water phase and chloroform phase is indicated in table 1. The radioactivity in the tissue residue is also shown in this table.

The chromatograms of the chloroform extracts with sufficient radioactivity for scanning or autoradiography showed one major spot with an Rf-value

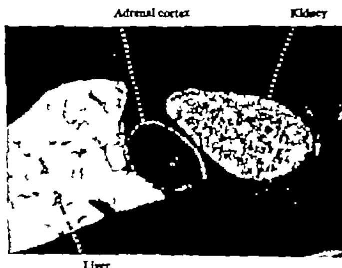


Fig. 3. Detail from an autoradiogram of a mouse 20 minutes after intravenous injection of ¹⁴C-4-aldosterone, showing the uptake in the glomerular zone of the adrenal cortex.

Table 1

Percentage of total radioactivity in the chloroform phase from different tissues at various times after intravenous injection of ^{14}C-4-aldosterone.

Time (min.)	Epididymis	Adrenal	Intestine	Testis	Kidney	Blood	Salivary gland	Liver	Bile	Ductus deferens
2	100 (2)	100 (4)	98	83 (4)	92	95	99 (7)	64	40	92 (23)
20	48	12	25 (9)	70 (2)	50 (5)	96 (45)	18 (19)	29 (2)	9	58

"Total" stands for the radioactivity in the combined chloroform and water extracts. The figures in brackets show the amounts of radioactivity exceeding 1% in the tissue residues.

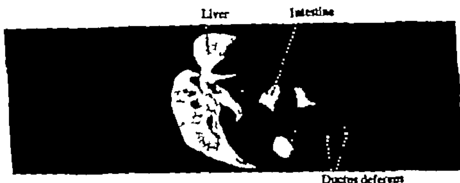


Fig. 4. Autoradiogram of a male mouse 60 minutes after intravenous injection of ^{14}C-4-aldosterone. High concentration of radioactivity in the bile ducts, intestinal contents (bile) and the ductus deferens.

corresponding to that of aldosterone (0.26–0.30) 2 minutes after injection. This was also the case for the epididymis, intestine, kidney liver and ductus deferens. The relative concentration in this spot was 83% in the extract obtained from the intestine and 93% from the kidney

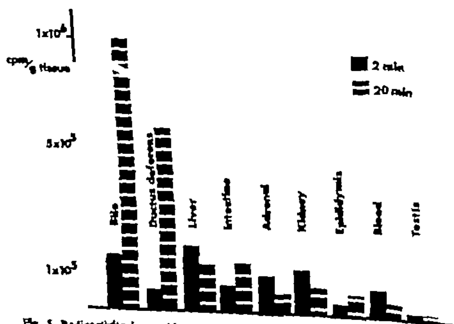


Fig. 5. Radioactivity in combined chloroform and water extracts of the bile, ductus deferens, liver intestine, adrenal, kidney epididymis, blood and testis, 2 and 20 minutes after the intravenous injection of ^{14}C-4-aldosterone.

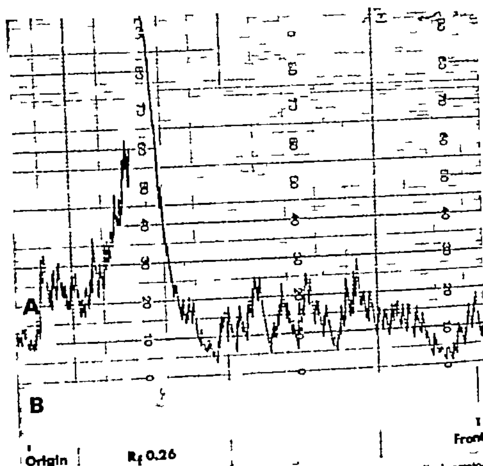


Fig. 6A. Diagram of radioactive thin layer chromatogram scanned in radiochromatogram scanner. Chloroform extract from ductus deferens 20 minutes after the injection of ^{14}C-4-aldosterone was chromatographed in chloroform-acetone-acetic acid (70:20:10).

Fig. 6B. Photograph of non-radioactive aldosterone from the same plate.

Twenty minutes after the injection, only about 50% of the radioactivity in the chloroform extract of the intestine showed a chromatographic behaviour indicative of aldosterone. In this organ the other 50% was represented by metabolites with Rf values of 0 14–0 18. The same general pattern as seen in the intestine was also registered after 20 minutes in the chloroform extract from the liver bile and epididymis. In the liver and bile some metabolites with Rf values 0.05–0 10 were also found. In the ductus deferens, however most of the radioactivity in the chloroform extract also corresponded to aldosterone 20 minutes after injection (fig. 6).

Discussion

The whole body autoradiographic distribution of ^{14}C- and ^{3}H-aldosterone agrees well with the distribution studies of labelled cortisone and cortisol by HANNGREN *et al.* (1964). Small differences could, however be observed and labelled aldosterone did not accumulate in the brown fat to the same extent as was found by these authors for cortisol and cortisone.

The rapid excretion of labelled aldosterone as shown previously (SULYA *et al.* 1963 MCCAA *et al.* 1964) was also observed in this autoradiographic study

The chromatographic and quantitative experiments indicated that 2 minutes after the injection of labelled aldosterone, most of the radioactivity was extractable by chloroform and corresponded to unchanged aldosterone. Twenty minutes after the injection, however most of the radioactivity was left in the water phase and/or in tissue residue, indicating metabolism and/or protein binding of the labelled aldosterone.

The mineralocorticoid effect of aldosterone may be triggered off by the formation of specific aldosterone receptor complexes in target cells (HERMAN *et al.* 1968). The presence of aldosterone-binding proteins in some tissues has recently been studied by SWANECK *et al.* (1969) who found considerable quantities of such proteins in the kidneys and duodenal mucosa and smaller quantities in the spleen, brain and liver A marked accumulation of labelled aldosterone was consistently found in the kidney in the present autoradiographic study but the accumulation in the intestinal mucosa was not as marked as in the kidney In the spleen and brain very small amounts of labelled aldosterone were found, which is in agreement with the findings of low aldosterone-binding ability in these organs. In the liver a very pronounced accumulation was found and this is probably due to the presence of steroid metabolizing enzyme systems. It is not yet known if the high concentrations of labelled aldosterone found in other organs, e.g. ductus deferens and parts of the caput epididymidis, are due to the presence of specific aldosterone receptors, indicating a true target, but this seems plausible.

It is thus very tempting to connect the accumulation of aldosterone in the caput epididymidis with the electrolyte changes observed there (CRABO & GUSTAFSSON 1964). Furthermore, since cortisol-using zones in the epididymis have been demonstrated histochemically (BAILLIE *et al.* 1966) the presence of the more potent mineralocorticoid aldosterone appears quite natural

The presence of labelled material in the glomerular zone of the adrenal cortex after the injection of labelled aldosterone is puzzling, but may be due to the equilibrium of labelled and non-labelled aldosterone possibly indicating a storage mechanism of aldosterone at its site of synthesis.

Acknowledgements

The authors are indebted to Knut and Alice Wallenbergs Stiftelse for general equipment for isotope research work.

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From the Institute of Pharmacology University of Oslo, Blindern, Oslo 3 Norway

The Action of Practolol on the Isolated Rat Atrium

By

Helge Refsum and Knut Landmark**

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Abstract. The β-receptor antagonism and the effects of practolol on the mechanical and electrical behaviour of isolated rat atria suspended in an organ bath have been investigated. Practolol produced a parallel shift to the right in the log. dose-response curves of isoprenaline with no decrease in the maximal response, indicating that the antagonism is competitive. The drug was found to exert positive inotropic and chronotropic effects in doses between 10^{-8} M and 10^{-6} M. Practolol 10^{-4} M did not change the threshold for excitation and the threshold for experimentally induced atrial fibrillation to any extent, neither did it prolong the effective refractory period. The results show that practolol has β-adrenergic receptor blocking properties as well as sympatomimetic properties. The results also indicate that practolol possesses no quinidine-like or local anaesthetic activity

Key-words: Practolol – isolated rat atria – sympatomimetic effects – isoprenaline – β-adrenergic receptor – antifibrillatory action.

Practolol (I.C.I. 50172) has been shown to produce a selective blockade of myocardial β-receptors in a dose range that produces no significant blockade of vascular β-receptors. The drug has also been reported to exert a relatively much weaker blockade of β-receptors in the trachea than of the cardiac β-receptors. (Barrett *et al.* 1968 Brick *et al.* 1968 Dunlop & Shanks 1968 Bristow *et al.* 1970).

Practolol has been used in clinical studies for more than three years and has been shown to increase exercise tolerance in patients with angina pectoris (Areskog & Adolfsson 1969 George *et al.* 1970 Sandler & Clayton 1970) control the rate in atrial and ventricular arrhythmias (Gibson *et al* 1968 Jewitt *et al.* 1969 Gent *et al* 1970) and lower elevated heart rate during exercise (Brick *et al* 1968 Gibson & Sowton 1968 Fitzgerald & Scales 1968 Shinebourne *et al* 1968) in thyrotoxicosis (Turner & Hill 1968) and during anaesthesia (Johnstone 1969)

Student Research Fellow Norwegian Research Council for Science and Humanities
** Research Fellow Norwegian Council on Cardiovascular Diseases

There is evidence that practolol is safer to use in patients with bronchial asthma (MacDonald & McNeill 1968, Sandler & Clayton 1970, Kerr & Patel 1970).

The aim of the present investigation was to study the mode of action of practolol by testing its effects on isolated rat atria. The main purpose was to evaluate whether the antifibrillatory action of practolol is due to a so-called quinidine-like effect, or whether it is a consequence of the β-receptor blocking activity of the drug.

Methods

Female, albino Wistar rats weighing about 200 g were used. The hearts were excised under ether anaesthesia, and the atria were quickly removed by dissection and suspended in a water-jacketed organ bath containing Ringer solution kept at 32. The Ringer solution had the following composition (ions in meq/l): Na^{+} 143.4, K^{+} 5.3, Ca^{++} 5.1, Mg^{++} 2.3, Cl^{-} 126.4, $H_2PO_4^{-}$ 2.4, HCO_3^{-} 25, SO_4^{--} 2.3. The solution also contained glucose 1.8 mg/ml, and it was bubbled with 95 % O_2 and 5 % CO_2. The pH was 7.4. A preload of 400 mg was put on the preparations and the contractile force was recorded isometrically with a Grass force-displacement transducer (FTO3C) connected to a Grass polygraph (Model 7WC12PA). Control experiments were performed at the end of a 30 min. period of equilibration.

In order to test the effect of practolol on the atria, the drug was added to the organ bath in concentrations ranging from 10^{-8} M to 10^{-4} M. Left and right spontaneously beating atria were used. The effect of each dose was measured 5 min. after the addition of the drug. The bath was then washed twice, and when stable values were again obtained, a new addition was made with a higher concentration of practolol. Log. dose-response curves were calculated for the inotropic and chronotropic effects of practolol.

Electrically stimulated left atria were used in order to investigate the inotropic effect of isoprenaline both in the absence and presence of practolol. The chronotropic effect of the drug under the same conditions was tested on *left and right* spontaneously beating atria. The atria were stimulated at a frequency of *180* per min. through bipolar platinum electrodes by square wave pulses of 0.5 msec. *duration* delivered by a Biotronic laboratory stimulator. Threshold voltage was used and measured on a Tektronix dual-beam oscilloscope, type 502 A, coupled in series with the atrium.

Changes in the amplitude of the contractions were measured over a range of doses of isoprenaline *from 10^{-10} M to 10^{-6} M. The contact time for isoprenaline,* administered at 10 min. intervals, was 2–3 min. The bath was washed twice between each addition of a drug. To determine the inotropic effect of isoprenaline in the presence of practolol, this drug added to the organ bath to give a final concentration of 10^{-6} M 4 min. before the administration of isoprenaline, the *effect of* which was measured after a further 2–3 min. The bath was washed twice between each addition of the drug.

Electrical threshold and maximum following frequency

In order to measure the threshold voltage required to drive the atria, the stimulus frequency was set about 10 per cent higher than the spontaneous atrial rate measured

at the end of the equilibration period. The duration of the pulses was 0.5 msec. The voltage was then gradually increased until the atria followed the imposed frequency. When measuring the maximum following frequency (m.f.f.), the stimulus intensity required to drive the atria was doubled. The rate of the stimulation was increased until the atria failed to follow i.e. when they began to drop beats. The duration of the pulses was kept constant.

After the control experiments had been carried out, practolol 10^{-4} M was added to the organ bath, and 10 and 20 min. later the test experiments were performed.

Threshold for fibrillation.

Left and right spontaneously beating atria were stimulated by square wave pulses of 2 msec. duration at a frequency of 1800 per min. The voltage was then increased until the contractions became incoordinated (defined as fibrillation). When the control experiments had been carried out, practolol 10^{-4} M was added to the organ bath and 10 min. later the test experiments were performed.

Calculations.

In order to compare the results, the values (for force and rate of contractions, electrical threshold and maximum following frequency) measured at the end of the equilibration period in each experiment were set at 100 per cent and used as reference.

Comparison of the results was made by using Student's t-test.

Drugs used.

Practolol HCl (I.C.I. 50172) and d'l-isoprenaline sulphate (A. G. Siegfried)

Results

Inotropic and chronotropic effects of practolol.

The atria resumed spontaneous contractions immediately after they were placed in the organ bath. The rate usually increased during the first 2–3 min., after which it almost remained unchanged throughout the equilibration period of 30 min. The contractile force gradually increased until a steady state was reached after about 20 min. The addition of practolol increased the force and the rate of contractions in a dose range from 10^{-8} M to 10^{-5} M. At the highest dose level (10^{-4} M) practolol did not have any significant inotropic or chronotropic effect (fig. 1). The force of contractions in the control period varied from 50 to 150 mg, the rate of contractions from 175 to 240 beats per min.

Effects of practolol on the inotropic and chronotropic effects of isoprenaline.

There was an almost parallel shift to the right and no fall in the maximum heights of the log. dose-response curves for isoprenaline in the presence of practolol 10^{-4} M. As shown in figs. 2 and 3 the antagonism was completely surmountable. The maximum inotropic and chronotropic effects of isoprenaline were virtually unchanged, although the log. dose-response

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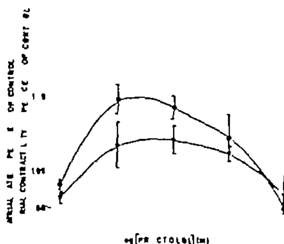


Fig. 1. Log. dose-response curves for the chronotropic and inotropic effects of practolol. Each value (mean value ± S.E.M.) represents the mean of five experiments and is calculated in per cent of the values obtained in the control period.

curves had been shifted to the right by approximately 1 log. unit. Practolol affected the inotropic and chronotropic effects of isoprenaline in a similar way.

Electrical threshold and maximum following frequency

The voltage required to stimulate the atria at a frequency 10 per cent higher than the spontaneous rate in the control period decreased slightly

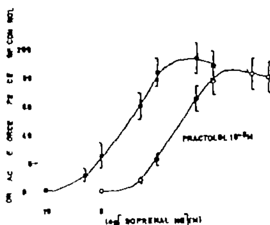


Fig. 2. Log. dose-response curves for the inotropic effect of isoprenaline in the absence and presence of practolol 10^{-6} M. Each value (mean ± S.E.M.) represents the mean of seven experiments and is calculated in per cent of the values obtained in the control period.



Fig. 3. Log. dose-response curves for the chronotropic effect of isoprenaline in the absence and presence of practolol 10^{-4} M. Each value (mean ± S.E.M.) represents the mean of five experiments and is calculated in per cent of the values obtained in the control period.

after exposure to practolol. However this decrease was not significantly different from the results obtained from control atria to which no drug was added ($0.05 < P < 0.10$ and $0.10 < P < 0.20$ after 10 and 20 min. respectively). The results are shown in table 1. The threshold before the addition of practolol varied between 0.80 V and 1.45 V in the different experimental groups.

At the end of the control period the maximum following frequency (m.f.f.) was approximately 560 beats per min. Above these values the atria failed to follow the imposed frequency (fig. 4). The administration of practolol caused a minimal increase in the m.f.f.. This increase was not significantly different from the results obtained from control atria to which no drug had been added ($0.10 < P < 0.20$ after 10 and 20 min. respectively). The results are shown in table 2.

Table 1

Threshold for electrical stimulation of atrial muscle in per cent of control. The statistical difference between the two groups is non-significant ($0.05 < P < 0.10$ and $0.10 < P < 0.20$ after 10 and 20 min. respectively).

Time (min.)	Practolol 10^{-4} M	No. of expts.	No drug	No. of expts.
10	93.1 ± 1.0	5	100.3 ± 2.8	8
20	93.8 ± 5.0	5	100.2 ± 3.2	8

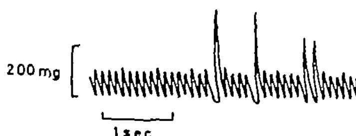


Fig. 4 Atrial preparation stimulated at a frequency of 600 per min. The tracing shows that the atria just fails to follow the imposed rate.

Threshold for fibrillation.

The threshold for fibrillation in the control period was 2.10 ± 0.12 V Practolol 10^{-4} M did not influence the threshold for experimentally induced atrial fibrillation to any significant extent (table 3).

Discussion

The mean log. dose-response curves for the inotropic and chronotropic effects of isoprenaline on isolated rat atria in the absence and presence of practolol, indicate a competitive β-receptor blocking action of practolol. This result is in agreement with studies of the action of practolol on the isolated rabbit atria (BRISTOW & GREEN 1970· BRISTOW *et al.* 1970 WALE 1970) The present study shows that practolol affects the inotropic and chronotropic log. dose-response curves for isoprenaline to the same extent, which supports the view that these effects are mediated by the same receptors (BLINKS 1967 BRISTOW & GREEN 1970)

The two main effects of a substance with antifibrillatory activity are to

Table 2

Maximum following frequency at which atrial muscle follows in response to electrical stimulation in per cent of control. The statistical difference between the two groups is non-significant ($0.10 < P < 0.20$ after 10 and 20 min. respectively).

Time (min.)	Practolol 10^{-4} M	No. of expts.	No drug	No. of expts.
10	99.3 ± 0.7	5	94.3 ± 2.4	8
20	99.3 ± 0.7	5	93.8 ± 2.9	8

Table 3

Threshold for experimentally induced atrial fibrillation before and after the addition of practolol.

Threshold in volts		No. of expts.
Control period	Practolol 10^{-4} M	
2.10 ± 0.12	2.06 ± 0.07	5

prolong the effective refractory period of cardiac muscle and to raise the threshold of excitation (SZEKERES & VAUGHAN WILLIAMS 1962) The refractory period is affected by several factors, including the driving frequency and the strength of the stimulus (DAWES & VANE 1956 TEXTER *et al.* 1967). Determinations of the maximum following frequency at which the atria will follow a stimulus, are therefore not an accurate test of the effective refractory period. However measurement of the maximum following frequency has proved to be of great practical value in the evaluation of synthetic substitutes for quinidine (DAWES 1946 & 1952), and as it is easy to perform, the method has been widely used as a screening standard for antifibrillatory action (HOLLAND 1957 VAUGHAN WILLIAMS & SZEKERES 1961 SEKIYA & VAUGHAN WILLIAMS 1963 DOHADWALLA *et al.* 1969· DAVIS 1970· LANDMARK 1971)

The present results indicate that practolol 10^{-4} M does not prolong the effective refractory period of isolated rat atria since it does not significantly influence the maximum following frequency at which the preparations respond to stimulation. Neither does practolol influence the threshold of excitation to any significant extent. In fact, the electrical threshold was slightly decreased and the maximum following frequency slightly increased after administration of the drug.

Fibrillation of atrial tissue can be produced experimentally by a combination of electrical stimulation with infusion of cholinergic substances (BURN *et al* 1955 HOLLAND & BRIGGS 1959), or by suitably timed electrical stimulation alone (DIPALMA & SCHULTS 1950· BROOKS *et al.* 1955 SZEKERES & LÉNÁRD 1960 LANDMARK 1971) The present investigation shows that practolol does not significantly influence the threshold for experimentally induced atrial fibrillation.

The above mentioned results indicate that practolol does not change the electrical activity of the rat atrium. DUNLOP & SHANKS (1968) have stated that practolol has no local anaesthetic activity and the present study seems to confirm this. However PAPP & VAUGHAN WILLIAMS (1969) have

found that practolol has marked quinidine-like effects on intracellular cardiac potentials (rabbit atria) at concentration above 6.61×10^{-4} M. On the other hand, this concentration has very little effect on the spontaneous frequency maximum following frequency electrical threshold, contractions and conduction velocity while 3.31×10^{-4} M of practolol influences these parameters to varying degrees. Membrane stabilizing drugs (like quinidine) have a negative inotropic and chronotropic effect (LANGSLET & ØYE 1970). The present results show that practolol, even at very high dose levels (10^{-4} M) does not have any negative inotropic or chronotropic effect on isolated rat atria. In contrast propranolol has marked negative inotropic and chronotropic effects at drug concentrations exceeding 10^{-6} M, and both these effects increase with increasing drug concentrations until arrest occurs at about 10^{-4} M (BLINKS 1967 NAYLER *et al.* 1969). It has been suggested by several investigators that a membrane (local anaesthetic) activity in a β-receptor blocking agent is clinically undesirable because of the depression of myocardial contractility (ELLIOTT & GORLIN 1966 GRANDJEAN 1967 LEVY 1968).

Both in the clinical and in the experimental field there has been some debate as to the relative importance of β-blocking properties and quinidine-like activity in explaining the antiarrhythmic effects of the β-receptor blocking agents. It appears however that β-receptor blockade is important for the reversal of catecholamine-induced arrhythmias, while a quinidine-like activity is essential for the reversal of arrhythmias due to cardiac glycosides (SOMANI & LUM 1965 LUCCHESI *et al.* 1969 DOLLERY *et al.* 1969) This view is supported by studies with practolol which has been found to be ineffective against ouabain induced arrhythmias, but effective in the prevention of ventricular fibrillation after the administration of chloroform and adrenaline (DUNLOP & SHANKS 1968). It has been suggested that practolol can be used in the differential diagnosis of cardiac arrhythmias, since it might be expected to suppress those caused by sympathetic overactivity but not those caused by digitalis (GIBSON *et al.* 1968).

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From the Department of Pharmacology Division of Clinical Pharmacology
and the Department of Pathology at Sabbatsberg Hospital, Karolinska Institutet
S-10401 Stockholm 60, Sweden

Oxidation and Glucuronidation of Certain Drugs in Various Subcellular Fractions of Rat Liver Binding of Desmethylimipramine and Hexobarbital to Cytochrome P-450 and Oxidation and Glucuronidation of Desmethylimipramine, Aminopyrine, *p*-Nitrophenol and 1 Naphthol

By

Christer von Bahr Eino Hietanen and Hans Glaumann

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Abstract 1 The plasma membranes and Golgi apparatus of rat liver were isolated. Total microsomes were sub-fractionated into rough microsomes, smooth I microsomes and smooth II microsomes. NADPH-cytochrome reductase activity cytochromes P-450 and b_5, the oxidation and glucuronidation of certain drugs were determined, and drug induced spectral changes were recorded. 2. Oxidation was measured with aminopyrine and desmethylimipramine as substrates. Glucuronidation of *p*-nitrophenol and of 1-napthol was measured. The "two-step reaction hydroxylation of desmethylimipramine followed by glucuronidation, was also studied *3* Only low activities of drug-oxidizing enzymes and UDP-glucuronyltransferase were found in the plasma and Golgi membranes. *4* Desmethylimipramine and hexobarbital induced the type I spectral change in the microsomal subfractions but not in the plasma and Golgi membranes. *5* Hydroxylation of desmethylimipramine occured at higher rates in the smooth-surfaced membranes than in the rough-surfaced membranes, whereas demethylation of aminopyrine was considerably lower in the smooth II microsomes than in rough and smooth I microsomes. *6*. *p*-Nitrophenol and 1-naphthol were glucuronidated to a somewhat higher extent in rough than in smooth I microsomes, but appreciably less in the smooth II membranes. However incubation of desmethylimipramine in the presence of cofactors required for both hydroxylation and glucuronidation resulted in glucuronide formation to about the same extent in all three microsomal subfractions. It is suggested *a*. that neither plasma membranes nor *Golgi* membranes play an important role in drug oxidation or glucuronidation reactions; *b*. that both rough and smooth I microsomes have high drug oxidizing and glucuronidating activities, while smooth II microsomes may be preferentially involved in the oxidation and glucuronidation of a limited number of substrates.

Key-words Microsomes – Golgi apparatus – plasma membranes – subcellular fractionation – drug hydroxylation glucuronidation cytochrome P-450.

Recipient of a Nordic Stipend from the Swedish Medical Research Council (B71 99P 3273-01). Present address: Department of Physiology University of Turku Turku 3 Finland.

A number of drugs, steroid hormones, fatty acids, and polycyclic hydrocarbons are oxidatively metabolized by enzyme components in the intracellular membranes of the liver (cf. ORRENIUS *et al.* 1968). The oxidized compounds are often subsequently conjugated with glucuronic acid by UDP-glucuronyltransferase (DUTTON 1966) before excretion into blood or bile.

Available data indicate that not only drug oxidation but also glucuronidation is catalyzed by enzyme systems primarily located in the intracellular membranes of the liver and that both these processes are dependent on the structural integrity of the membranes (IMAI & SATO 1960 GRAHAM & WOOD 1969). However the localization of the enzyme systems among different cytomembranes is not fully known, except for rough and total smooth microsomes (GRAM *et al* 1968 MULDER 1970) It recently became possible to isolate subfractions of smooth microsomes and reasonably pure plasma membranes (cf. BENEDETTI & EMMELOT 1968) and Golgi elements (MORRÉ *et al.* 1970 GLAUMANN & ERICSSON 1970). Since the Golgi apparatus is often located near the bile canaliculi in the hepatocyte and a large number of endogenous and exogenous compounds are preferentially excreted into the bile in a conjugated form, it might well be inferred theoretically that Golgi membranes participate in the conjugation reactions and in excretion.

We have investigated the abilities of various intracellular membranes to oxidize and glucuronidate drugs. Rough microsomes, smooth I and II microsomes, plasma membranes and Golgi elements were isolated, and the distribution of the drug metabolizing enzymes was investigated by means of the following reactions (I) hydroxylation in a one-step reaction of the highly lipophilic drug, desmethylimipramine (DMI), which is excreted metabolized as well as unchanged into the bile (VON BAHR & BOROÅ 1971) and oxidation of a less lipophilic compound, aminopyrine· (II) hydroxylation and subsequent glucuronidation of DMI in a "two-step reaction" (VON BAHR 1970· VON BAHR & BERTILSSON 1971) in which the two enzyme sequences cooperate, as they do *in vivo* to yield more polar metabolites, (III) glucuronidation of *p*-nitrophenol and 1-naphthol in digitonin treated membranes· In addition NADPH-cytochrome *c* reductase, cytochrome P-450 and drug induced spectral changes were determined.

Material and Methods

Animals

Male Sprague-Dawley rats weighing 200–250 g were used. They were starved for 16 hours before sacrifice.

Subfractionation.

The microsomes were sub-fractionated as described previously (DALLNER 1963, GLAUMANN & DALLNER 1968) with certain modifications. A 25% homogenate of liver in 0.25

M sucrose was centrifuged at 10,000 g for 20 min. Sufficient 1 M-CsCl was added to the supernatant to give a final concentration of 13 mM. 8 ml of this supernatant was layered over 3.5 ml of 1.30 M sucrose – 0.013 M-CsCl. After centrifugation at 250,000 g for 60 min. (Christ Omega II ultracentrifuge, rotor 60, tube angl 34°), the reddish, clear upper phase was removed with a pipette provided with a rubber aspirator and discarded. The entire fluffy double layer at the gradient boundary (about 2 ml) was collected together with about 0.5 ml of the 1.30 M clear sucrose solution. The fluffy layers from two centrifuge tubes were combined, slowly diluted with distilled water to 8 ml, mixed with 1 M-$MgCl_2$ to give a final concentration of 7 mM, and layered over 3.5 ml of 1.15 M sucrose – 0.007 M-$MgCl_2$. After centrifugation at 250,000 g for 30 min., the clear upper phase was removed and discarded. The thin fluffy layer at the gradient boundary was collected, diluted with 0.25 M sucrose, and re-centrifuged at 105,000 g for 120 min. After re-suspension of the pellet in 0.25 M sucrose, we obtained the fraction called *smooth II microsomes*.

The 1.15 M sucrose of the gradient containing Mg was decanted, and, after resuspension of the pellet, the fraction was called *smooth I microsomes*.

Since the pellet obtained in the first gradient centrifugation in the presence of Cs sometimes had a loose surface, the 1.30 M sucrose was not completely removed: a few drops of the sucrose were left behind. Distilled water was added to bring the sucrose concentration to 0.25 M, and the pellet, called *rough microsomes* was re-suspended.

Plasma membranes were separated by the method of Coleman *et al.* (1967).

The isolation of a subcellular fraction composed primarily of Golgi elements was performed according to Morré *et al.* (1970) with certain modifications (Glaumann & Ericsson 1970). For this purpose, 50 g of liver from exsanguinated rats were used. The livers were minced thoroughly and rinsed in 0.50 M sucrose-5 mM $MgCl_2$ solution. No buffer or dextran was included in any of the media used in this procedure. Mild homogenization was obtained with a Teflon-glass homogenizer (chamber clearance, 0.006–0.009 inch) at low speed (100 r.p.m.) with only two complete strokes. After dilution to 90 ml with 0.50 M sucrose –5 mM $MgCl_2$ solution, the homogenate was centrifuged at 3,000 g for 20 min. in a Christ Omega II ultracentrifuge (SW 27). The white upper third of the pellet was diluted to 7 ml with 0.25 M sucrose, layered over 22 ml of 1.25 M sucrose solution, and centrifuged at 60,000 g for 60 min. (SW 27). The white band at the interphase was collected and sedimented t 3,000 g for 30 min. The pellet was washed twice in 0.25 M sucrose solution by gentle suspension in a Teflon glass homogenizer re-centrifuged, and designated as the *Golgi fraction*. The composition of this fraction was checked by electron microscopy and the results were found to agree with previous findings (Glaumann & Ericsson 1970).

Enzyme assays.

The hydroxylation of ^3H-DMI was measured in a system containing 2 mg of protein of the membrane fraction, 50 mM Tris-Cl buffer (pH 7.5), 5 mM-$MgCl_2$, 1 mM-NADP 5 mM-DL-isocitrate, 0.005 mM-$MnCl_2$, and 0.4 I.u. pig heart isocitric dehydrogenase (see below) in final volume of 2 ml. Incubation was performed at 37 and the reaction was stopped by freezing. Uridine-5'-diphosphoglucuronic acid (UDPGA, 1 mM) was added, when it was necessary that the hydroxylated metabolites should be glucuronidated. Analysis of the metabolites was performed as described elsewhere (von Bahr & Borgå 1971).

The incubation system of Hänninen (1968) was used in order to measure the glucuronidation of *p*-nitrophenol. It contained 0.05 ml of digitonl treated membranes (see below), and 0.33 mM *p*-nitrophenol, 10 mM sodium-EDTA (Titriplex III), and 7.5 mM

UDPGA dissolved in 0.1 ml 0.5 M NaH_2PO_4 (pH 7.5). Incubation (0.15 ml) was performed for 0, 10, and 20 min. at 37° The reaction was stopped with 0.9 ml of 2.8 per cent TCA. The mixture was then pelleted and 0.1 ml of 5 M NaOH was added to the supernatant. Its optical density was measured at 400 nm and compared with identical samples obtained from an incubation mixture without UDPGA. The decrease in optical density reflects the disappearance of unconjugated *p*-nitrophenol (ISSELBACHER 1956).

The glucuronidation of 1-naphthol was assayed in an identical incubation system containing 0.1 mM 1-naphthol. Incubation was stopped with 2.0 ml of 0.05 M-NaOH. The fluorescence of this mixture was measured in an Aminco-Bowman spectrophotofluorometer. The excitation wavelength was 333 nm and the emission wavelength 460 nm (VERITY *et al.* 1964). The fluorescence of the samples was compared with that of identical samples without UDPGA.

The levels of cytochrome P-450 and cytochrome b_5, the NADPH-cytochrome c reductase and the aminopyrine demethylase activities were measured as before (GLAUMANN 1970), as were the drug induced spectral changes (VON BAHR & ORRENIUS 1971).

Other methods.

One and one half parts of a 1 per cent aqueous solution of digitonin were added to one part of subcellular fraction (∼10 mg protein/ml) in 0.25 M sucrose solution, and this was followed by incubation for 30 min. at 4° (HÄNNINEN 1968). The non-solubilized membranes were sedimented at 105,000 g for 60 min. and then used for incubation.

Protein was determined by the method of LOWRY *et al.* (1951), and phospholipids and cholesterol by the methods previously described (GLAUMANN & DALLNER 1968).

Chemicals.

^{3}H DMI-HCl (13.0 mCi/mmol) labelled in the 10- and 11-positions was used (kindly donated by AB Leo, Sweden). The radiochemical purity was ≥ 99 per cent as tested in three thin layer chromatographic systems (VON BAHR & BORGÅ 1971). All the other chemicals were commercial products of reagent grade. Pig heart isocitric dehydrogenase was obtained from C. F. Boehringer & Soehne, Mannheim, West-Germany.

Results

Table 1 shows the results of quantitative estimations of the protein, phospholipid, and cholesterol contents of the microsomal subfractions, the plasma membranes, and the Golgi fraction. The bulk of the total microsomal phospholipids was recovered in the rough subfraction, about 30 per cent and 10 per cent appearing in the smooth I and smooth II subfractions, respectively. The combined Golgi and plasma membrane fractions contained about 10 per cent of the total microsomal protein. It should be pointed out however that, whereas the recovery of microsomes from liver homogenates is about 50 per cent (GLAUMANN 1970), reliable estimates of the recoveries of plasma membranes and Golgi profiles are not possible with the existing methods of isolation. The smooth II membranes and particularly the plasma membranes, were characterized by high concentrations of cholesterol as compared with

Table 1

Protein, phospholipids and cholesterol in various subcellular fractions of rat liver Subfractionation was performed as described in Material and Methods. The values are means of 4 experiments ± S.D., mg/g of liver (wet weight)

	Protein	PLP[1]	Cholesterol	Cholesterol / PLP
Total microsomes	18.6 ± 2.3	6.1 ± 0.8	0.53 ± 0.05	0.09
Rough microsomes	9.7 ± 1.1	2.9 ± 0.4	0.20 ± 0.03	0.07
Smooth I microsomes	5.4 ± 0.6	1.9 ± 0.2	0.21 ± 0.02	0.11
Smooth II microsomes	1.1 ± 0.1	0.41 ± 0.05	0.062 ± 0.01	0.15
Golgi fraction	0.69 ± 0.1	0.21 ± 0.02	0.021 ± 0.003	0.10
Plasma membranes	1.1 ± 0.1	0.39 ± 0.05	0.10 ± 0.02	0.26

[1] PLP phospholipids.

the rough microsomes and Golgi membranes. A high content of cholesterol in plasma membranes has been observed by several investigators (cf. BENEDETTI & EMMELOT 1968).

Table 2 demonstrates the distribution pattern of NADPH-cytochrome *c* reductase, cytochrome P-450, aminopyrine demethylase activity and cytochrome b_5 among the fractions. The levels of these enzyme components in the Golgi and plasma membrane fractions ranged from 12–25 per cent of those found in smooth I microsomes. The smooth II subfraction displayed low activities of NADPH-cytochrome *c* reductase and aminopyrine demethylase, but the amounts of cytochromes P-450 and b_5 were almost equally distributed among all of the microsomal subfractions.

Many compounds can induce characteristic absorbance changes in liver microsomes. This is believed to be related to the interaction with cytochrome P-450 and the substrate (SCHENKMAN *et al.* 1967). To investigate such substrate binding in the various membranes, we studied the spectral changes induced by DMI and hexobarbital (enhexymalum NFN). The latter drug was chosen because it is reported to elicit a typical "type I spectrum" (SCHENKMAN *et al.* 1967) which is characterized by a peak at about 385 nm and a trough at about 425 nm in the difference spectrum of the microsomal suspension. Both DMI and hexobarbital induced the type I change in total microsomes and in smooth II microsomes (fig. 1). No significant change occurred either in the plasma membranes or in the Golgi membranes (not shown in figure).

The apparent K_s-value (SCHENKMAN *et al.* 1967) is generally believed to be a measure of substrate affinity for cytochrome P-450. It can be calculated similarly to apparent K_m values, namely by plotting 1/Δ O.D. on the or

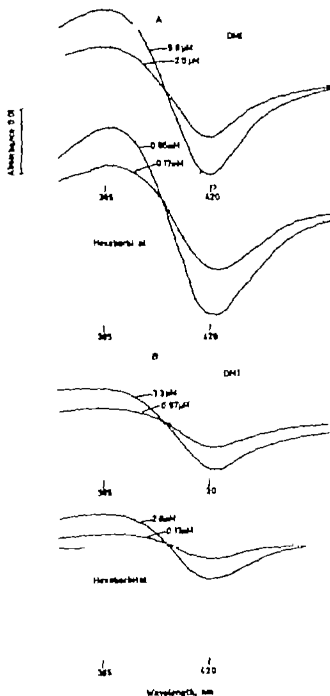


Fig. 1. Type I spectral changes induced by desmethylimipramine (DMI) and hexobarbital in total (A) and smooth II (B) microsomes. Microsomal suspension was added to a pair of cuvettes. The drugs were added to the sample cuvette and the absorbancy difference spectra were scanned. The concentration of protein was 3.6 mg/ml with the total and 1.2 with the smooth II microsomes.

Table 2

Enzyme distribution in various subcellular fractions.
Subfractionating was performed as in table 1. The values are means of 4 experiments ± S.D.

Enzyme	Rough microsomes	*Smooth I* microsomes	Smooth II microsomes	Golgi fraction	Plasma *membranes*
NADPH-cytochrome *c* reductase[1]	0.047 ± 0.007	0.059 ± 0.008	0.017 ± 0.003	0.016 ± 0.003	0.010 ± 0.001
Cytochrome P-450[2]	0.52 ± 0.06	0.60 ± 0.07	0.40 ± 0.05	0.07 ± 0.03	0.10 ± 0.02
Aminopyrine demethylase[3]	3.1 ± 0.7	3.6 ± 0.8	1.2 ± 0.2	*0.9 ± 0.4*	0.9 ± 0.4
Cytochrome b_5[2]	0.48 ± 0.08	0.57 ± 0.10	0.40 ± 0.07	0.16 ± 0.02	0.16 ± 0.05

1 µmol NADPH ox./min. × mg protein.
2 nmol/mg protein.
3 nmol formaldehyde/min. × mg protein.

Table 3

Desmethylimipramine- and hexobarbital-induced spectral shifts in total and smooth II microsomes.
Microsomal suspension was added to a pair of cuvettes. Increasing amounts of drug were then added to the sample cuvette. The values of K_s and $O.D._{max}$ were calculated on the difference in optical density between 385 and 420 nm. The K_s-value was calculated in analogy with calculation of K_m values by plotting $1/\Delta$ O.D against $1/(S)$.

		K_s (μM)		Δ O.D max. per mg protein		per nmol P-450	
	Exp.	DMI	Hexobarbital	DMI	Hexobarbital	DMI	Hexobarbital
Total microsomes	1	3.8	530	0.0026	0.0038	0.0055	0.0079
	2	3.3	330	0.0037	0.0050	0.0057	0.0077
Smooth II microsomes	1	1.2	380	0.0041	0.0033	0.012	0.0099
	2	1.4	330	0.0038	0.0048	0.010	0.013

dinate and 1/(S) on the abscissa. As shown in table 3 the apparent K_m-value for DMI was lower in the smooth II microsomes than in the total microsomes. On the other hand, the values for hexobarbital were similar in these fractions.

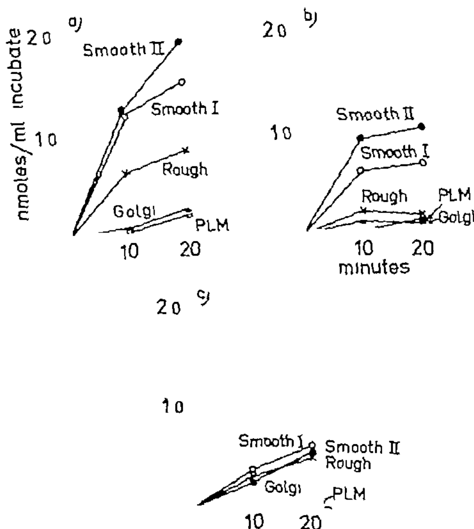


Fig. 2. Hydroxylation and subsequent glucuronidation of desmethylimipramine (DMI) in various subcellular fractions. a. Formation of hydroxylated, unconjugated metabolites in the absence of UDPGA ("one-step reaction"). b. Accumulation of the unconjugated, hydroxylated intermediate metabolites in the presence of UDPGA (10^{-3}M) in the "two-step reaction". c. Formation of glucuronides from DMI in the "two-step reaction". The initial concentration of DMI was 2.7×10^{-5}M. The incubation mixture contained 1 mg protein/ml. PLM, plasma membranes. The figure shows a single typical experiment. The same pattern was found in 3 other experiments.

The rate of hydroxylation of DMI in different subcellular membranes is shown in fig. 2a. All three microsomal subfractions, and especially the smooth-surfaced varieties, showed considerable activities in contrast to the plasma membranes and Golgi membranes, which exhibited only low capacities to hydroxylate DMI. Fig. 2b shows the accumulation of the hydroxylated intermediate metabolites of DMI as a function of time in the two-step reaction. DMI was incubated in the presence of cofactors for both hydroxylation and glucuronidation. The pattern is similar to that seen in fig. 2a except for the rough microsomes, where the accumulation was almost as low as in the plasma membranes and Golgi fraction. This was due to a rapid subsequent metabolism of the hydroxylated DMI to the corresponding glucuronide, as is shown in fig. 2c, which demonstrates an equal rate of glucuronidation from DMI for the three microsomal subfractions. Thus the ratio between the amounts of formed glucuronides (fig. 2c) and the accumulated, unconjugated but hydroxylated metabolites (fig. 2b) was higher in rough microsomes than in the smooth I and, especially in the smooth II varieties. Only negligible amounts of glucuronides of DMI were produced when plasma or Golgi membranes were incubated.

Fig. 3 summarizes the distribution of glucuronidation activities with *p*-nitrophenol and 1-naphthol as substrates in rough- and smooth-surfaced microsomes, plasma membranes, and Golgi elements. All the fractions were pre-incubated with digitonin, which is known to produce a multifold increase of UDP-glucuronyltransferase activity probably because of an increased availability of the substrate and/or UDPGA to the enzyme (HÄNNINEN & PUUKKA 1970). Rough and smooth I microsomes displayed high levels of conjugation as compared with smooth II microsomes, the Golgi apparatus, and

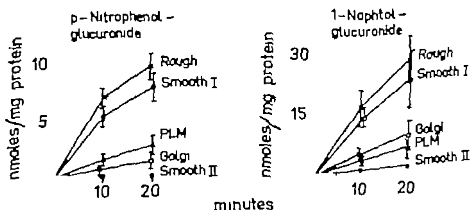


Fig. 3. Distribution of UDP-glucuronyltransferase in various subcellular fractions. In (a) *p*-nitrophenol, and in (b) 1-naphthol was used as substrate. Experiments were performed as described in *Material and Methods*. The values are means of 4 experiments ± S.E.M.

plasma membranes. Glucuronidation occurred somewhat more rapidly in the rough than in the smooth I microsomes.

Discussion

Although it is known that drug metabolism, including glucuronidation re actions, occurs in the endoplasmic reticulum, little is known about the distribution pattern among various well characterized submicrosomal fractions and practically nothing as to whether UDP-glucuronyltransferase activities are also present in plasma membranes and membranes of the Golgi apparatus. In order to investigate this, various intracellular membranes were isolated, and the oxidation and glucuronide formation of certain drugs were measured.

DMI was hydroxylated and aminopyrine demethylated more rapidly in the rough and smooth I microsomes than in the plasma and Golgi membrane fractions.

The slow rate of oxidation in the plasma and Golgi membranes is probably due to a minor contamination with membranes of the endoplasmic reticulum. Thus neither the plasma membranes nor the Golgi apparatus would seem to play an important role in drug oxidation. This is further supported by the low levels of NADPH-cytochrome c reductase activity and of cytochrome P-450 in these membranes (table 2), and also by the lack of spectral changes with DMI and hexobarbital.

The formation of glucuronides from DMI, *p*-nitrophenol, and 1-naphthol occurred more rapidly in the rough and smooth I microsomes than in the plasma and Golgi membranes. The oxidation and glucuronidation activities thus seem to be quite similarly distributed among microsomes on the one hand and among the plasma and Golgi membranes on the other

The smooth II microsomal fraction may be of special interest, since aminopyrine demethylase and NADPH-cytochrome *c* reductase activities were considerably lower in these membranes than in the smooth I and rough varieties. In contrast, oxidation of DMI was somewhat concentrated in the smooth II membranes, which agrees with the low K_s-value and high Δ O.D.$_{max}$/nmol P-450 value in this fraction (table 3) The distribution pattern of hydroxylation of DMI and demethylation of aminopyrine in smooth II microsomes indicates that these membranes participate mainly in the oxidation of certain substrates. Another alternative would be that the known high affinity of DMI to cytochrome P-450 (von Bahr & Orrenius 1971), is valid for all types of microsomal membranes, whereas differences in permeability or affinity as regards aminopyrine could exist among the microsomal subfractions. The varying content of cholesterol in the different subfractions (table 1) may be important for the permeability of drugs, since cholesterol is known to

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From the Department of Pharmacology University of Oslo, Blindern, Oslo 3, Norway

α Adrenergic Receptor Stimulation with Phenylephrine in Rabbit Fundus Muscle Exposed to Carbachol

By

J. F. W. Haffner

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Abstract: The effects of carbachol and phenylephrine on circular and longitudinal muscle from the fundus of the rabbit stomach have been studied. Carbachol ($2 \times 10^{-8} - 5 \times 10^{-6}$ M) produced an increase in tone in all the strips. The maximum response in the circular strips was approximately 4 times greater than that in the longitudinal strips. The response to phenylephrine depended on the existing tone of the muscl when the drug was added. In *inactive* strips, phenylephrine ($2 \times 10^{-7} - 10^{-4}$ M) always increased the tone. I *moderately contracted* (10^{-7} M carbachol) longitudinal strips, the response was biphasic – a transient relaxation preceded sustained contraction – while in the circular strips it was purely excitatory. In *strongly contracted* ($5 \times 10^{-7} - 2 \times 10^{-6}$ M carbachol) strips, phenylephrine caused a small transient relaxation, irrespective of whether the strips were cut circularly or longitudinally. The results emphasize the importance of defining the existing tone when responses to catecholamines are studied.

Key-words. α-Adrenergic receptors – gastric motility – gastric smooth muscle.

In a previous study (Haffner 1971) it was found that the activity in muscle from the fundus of the rabbit stomach was mainly tonic (= slow reversible changes in tension), whereas the activity in muscle from the antrum was mainly phasic (= rhythmic contractions). Preparations from the fundus and antrum are therefore best studied separately. The slow sustained alterations in tone make the fundus preparations suitable for studies of drug actions, and these preparations have therefore been used in the present study.

In the previous study the response to adrenergic agents appeared to depend on the existing tone when the drugs were added, but as the tone was allowed to vary spontaneously it was difficult to judge how often the different responses occurred, and the exact relationship between the existing tone and the drug response. In the present investigation the effect of phenylephrine on rabbit fundus strips exposed to varying concentrations of carbachol has been studied in order to determine the relationship between the existing tone and the response to adrenergic α-receptor stimulation.

Material and Methods

22 female white rabbits, weighing from 2 to 4 kg were killed by a blow on the neck and then bled by cutting the carotid arteries. The stomach was removed and 4 full-thickness (< 1 mm) muscle strips 2–3 mm wide and 2 3 cm long were cut either circularly or longitudinally from the anterior wall of the fundus, leaving the mucosa intact. The strips were taken approximately half way between the level of the cardiac sphincter and the top of the stomach. They were tied to a glass holder and submerged in an organ bath containing 50 ml modified Krebs solution at 37 aerated with 95% O_2 and 5% CO_2. The Krebs solution contained (mM) Na 136.9 K 5.9 Ca 1.25 (NB! half the concentration used in the previous study (HAFFNER 1971)), Mg 1.2, HCO_3 15.5 H_2PO_4 1.2, Cl^- 133.6 and glucose 11.5. The free end of the strip was tied to an isometric force-displacement transducer (Grass FT03) connected to a Grass polygraph. The transducer was adjusted until the resting tension (= stretch) was approximately 500 mg. The low degree of stretch and the reduction in calcium content were used in order to avoid spontaneous development of tone. The preparations were allowed to equilibrate for at least 30 minutes before the drug responses were tested.

Carbachol and phenylephrine were added in amounts which gave bath concentrations of 2×10^{-8} to 5×10^{-6} M and 2×10^{-7} to 10^{-4} M respectively The drugs were left in the bath until a maximal effect had been attained, usually 5 minutes, after which the bath was completely emptied and refilled. Dose-response curves were worked out for the effect of carbachol on circular and longitudinal strips. The increases in tone produced by different concentrations of carbachol were found to be reproducible in individual preparations. Dose response curves for phenylephrine could therefore be worked out in completely relaxed strips, and also in preparations which were contracting in response to 10^{-7} 5×10^{-7} and 2×10^{-6} M carbachol. The phenylephrine was added after the carbachol-induced contraction had reached a stable level, and never less than 5 minutes after the addition of carbachol. Only one concentration of phenylephrine was tested at a time. Completely fresh strips were used for the dose-response curves for carbachol and phenylephrine in relaxed circular and longitudinal strips while the dose-response curves for phenylephrine in contracted strips were carried out on preparations in which the *qualitative effect of phenylephrine had first been tested in the* presence of varying concentrations of carbachol.

The drugs used were: Carbacholine chloride (= carbacholinum chloridum NFN) and phenylephrine HCl (metaoxedrinum NFN).

Results

No spontaneous activity or tone was observed in the strips under the experimental conditions used in this investigation. As in the previous study (HAFFNER 1971) the effect of the drugs on the fundus strips were seen as alterations in tone, phasic activity occurred very rarely and when present, the amplitude of the contractions was small compared with the alterations in tone.

Carbachol.

Carbachol in concentrations from 10^{-7} (threshold $2 \times 10^{-8} - 5 \times 10^{-7}$) to 5×10^{-6} M gave a dose dependent sustained increase in tension. The same

effect was obtained with acetylcholine, but carbachol was chosen as stimulating agent in this investigation because it generally gives a more prolonged effect than acetylcholine.

The qualitative effect of carbachol was the same in all the strips but the magnitude of contraction was much greater in the circular than in the longitudinal preparations. Fig. 1 (left) shows dose-responce curves for carbachol, and it can be seen that the circular preparations develop 4 times as much tension as the longitudinal in response to 10^{-6} M carbachol. The threshold is the same, but the maximum effective doses differ in these different preparations (longitudinal 10^{-5} M, circular 5×10^{-6} M).

Phenylephrine.

The effect of phenylephrine depended on the existing state of contracture when the drug was added (fig. 2)

a) at zero tone – i.e. in the absence of carbachol – phenylephrine produced a dose-dependent sustained increase in tension, both in the circular and longitudinal strips. This effect was similar to that of carbachol, but the latter drug gave a maximum effect which was far greater than the maximum effect of phenylephrine. In fig. 1 (right) the dose-response curves for phenylephrine in circular and longitudinal strips are compared. It can

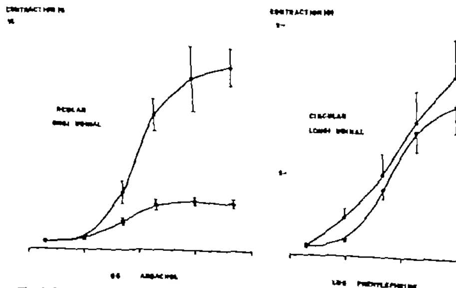
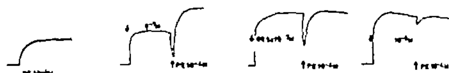


Fig. 1. Log dose-response curves for carbachol (left) and phenylephrine (right) in completely relaxed circular and longitudinal rabbit fundus strips. Each curve represents the mean (± S.E.M.) of eight experiments. Note that the scale of contraction for carbachol is ten times that of phenylephrine

LONGITUDINAL FUNDUS STRIP



CIRCULAR FUNDUS STRIP

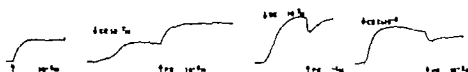
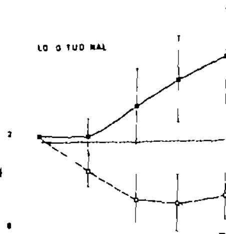


Fig. 2. The *qualitative* response to phenylephrine (PE) in rabbit fundus strips. Note the difference in the effect of PE on longitudinal and circular strips which are contracted by 10^{-7} M carbachol (CC).
(The scale of contraction is not the same for the various responses, for quantitative effects, see figs. 1, 3 and 4)



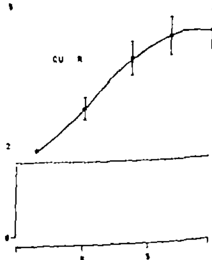


Fig. 3 Log dose-response curves for phenylephrine in fundus strips which were contracted by 10^{-7} M carbachol (dotted line indicates mean tension due to carbachol). In the longitudinal strips the effect was biphasic, an inhibitory effect (open squares) preceding contraction (filled squares). In the circular strips the effect was purely excitatory (filled circles).

be seen that in contrast to the great difference in maximal contractile response to carbachol, only a slight difference is found in the magnitude of the response to phenylephrine in muscle from the two layers.

b) *With moderately increased tone* in the preparations – due to the addition of 10^{-7} M carbachol – a qualitative difference between the response in the circular and longitudinal strips became apparent. The response to phenylephrine became biphasic in longitudinal strips a brief relaxation preceded a prolonged contraction, while it remained purely excitatory in the circular strips. Both components of the response in the longitudinal strips depended on the dose, as did the excitatory effect of phenylephrine in the circular strips (fig. 3), but the maximum relaxing effect of phenylephrine in the longitudinal strips occurred at lower concentrations than the maximum contractile effect.

c) *With high existing tone* in the strips – due to addition of 5×10^{-7} or 2×10^{-6} M carbachol – different results were obtained low concentrations (less than 5×10^{-6} M) of phenylephrine had no effect, but high concentrations gave a small transient relaxation both in the circular and longitudinal preparations (see fig. 4).

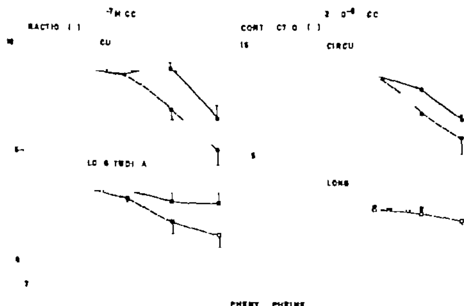


Fig. 4. Log dose-response curves for phenylephrine in fundus strips contracted by 5×10^{-7} M (left) and 2×10^{-6} M (right) carbachol (CC). The response had a biphasic character in both circular and longitudinal strips; after the initial relaxation (open circles and squares) the tension rapidly increased gain (filled circles and squares) approaching the initial tension. Note difference in scales of contraction.

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From the Department of Pharmacology University of Copenhagen,
20 Juliane Maries Vej,
DK 2100 Copenhagen Ø Denmark

In Vitro Studies on the Uptake of ^{14}C Labelled Tetraethylammonium in Mouse Kidney*

By

Jan Holm

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Abstract Tetraethylammonium (TEA) uptake was studied in mouse kidney slices. The slices were incubated in Krebs-Ringer bicarbonate medium (37 pH 7 4) containing ^{14}C TEA, and the ^{14}C-slice-to-medium (S/M) concentration ratio was measured. TEA (2 μM) was rapidly accumulated in oxygen-carbon dioxide (95:5) atmosphere, and a steady state with S/M ratio approximating 20 was obtained within ½ hr The radioactive material extracted corresponded chromatographically to authentic TEA. The S/M ratio was significantly reduced in the presence of metabolic inhibitors or various quaternary and tertiary amines. In nitrogen-carbon dioxide (95:5) atmosphere the S/M ratio (2 μM) reached a constant value around 1 within ½ hr Incubation at 19 markedly depressed the 3 min. S/M ratio, but had no effect on the steady state S/M ratio (½ hr) Under anaerobic conditions TEA uptake (mmol/kg tissue· ½ hr incubation) was directly proportional to the external TEA concentration. The aerobic uptake consisted of two components: a saturable one (maximum capacity 2 mmol/kg; half saturation concentration 140 μM) and one that increased proportionally with the external concentration. It is concluded that TEA is taken up against a concentration gradient by a saturable, energy dependent process which is inhibited by structural analogues.

Key words: Tetraethylammonium – transport – mouse kidney slices – *in vitro*.

Tetraethylammonium (TEA) is conventionally used as a marker substance for the organic cation secretion mechanism in the kidney This monoquaternary ammonium compound was shown to be rapidly secreted by kidney tubules in the dog and chicken (RENNICK *et al.* 1954) and to be actively accumulated in kidney slices of both species (FARAH & RENNICK 1956 FARAH *et al.* 1957 MCISAAC 1969).

A preliminary report on this study was presented at the XXV Scandinavian Pharmacological Meeting, Copenhagen 1971

In association with studies on the renal transport of quaternary ammonium compounds, we have shown that mouse kidney slices accumulate decamethonium by a saturable, energy dependent process (HOLM 1970a). Recent studies have indicated that decamethonium and TEA share a common transport mechanism in mouse kidney slices (HOLM 1971a)

We therefore found it of interest to study the uptake process of TEA in mouse kidney in order to compare it with that of decamethonium. The present experiments were performed in order to establish whether the uptake of ^{14}C TEA by mouse kidney slices follows saturation kinetics, requires energy and is inhibited by structural analogues (compounds possessing positively charged nitrogen atoms at physiological pH). The possibility of metabolic transformation of ^{14}C TEA was investigated by a paper chromatographic method (BRØEN CHRISTENSEN & HOLM 1969).

Materials and Methods

Tetraethyl 1-C^{14} ammonium bromide (specific activity 3.0 mci/mM) was supplied by New England Nuclear Corp., Boston, U.S.A.

The following non-labelled compounds were used, tetraethylammonium bromide (Ble & Berntsen, Denmark) N1-methylnicotinamide iodide (Sigma, St. Louis, U.S.A), carbamoylcholine chloride (Ph.Nord. 1963), neostigmine bromide (Ph.Nord. 1963), physostigmine salicylate (Ph.Nord. 1963) atropine sulfate (Ph.Nord. 1963), d-tubocurarine dichloride (Abbott) hemicholinium-3 (Aldrich, Chemical Co. Inc., U.S.A.), choline chloride (Merck), hexamethonium dibromide (May & Baker Ltd.), decamethonium diiodide (Koch-Light Laboratories Ltd., England) ouabain (g-strophantin, Ph.Nord. 1963), 2.4 (a)-dinitrophenol (Merck) and iodoacetic acid (Merck).

Experimental procedure

Slices of mouse kidneys were prepared and used as previously described (HOLM 1970a). Male albino mice of a single strain (NMRI) with a body weight of 23–32 g were decapitated and bled. Four slices were cut from each kidney with a razor blade. Each of the experiments recorded in table 1 and 2 was carried out as a paired comparison with the kidney tissue from two animals. Eight slices (total wet weight: 150–200 mg) from two kidneys (one kidney from each animal) were placed in a test tube, which contained 20 ml Krebs-Ringer bicarbonate solution with glucose (11 mM/l). This procedure made it possible to incubate kidney tissue from each animal (distributed in two test tubes) simultaneously under different experimental conditions (with or without treatment) Each animal thus served as its own control, method of paired comparison (BRADFORD HILL 1966). In the remainder of the experiments each test tube contained only kidney slices from the same animal (both kidneys)

Incubations were carried out by shaking (60 oscillations/min.) the test tubes at 37° (pH 7.4) and bubbling a 5:95 v/v% mixture of either carbon dioxide-oxygen or carbon dioxide-nitrogen through the medium. The slices were submerged in the salt solution 10 minutes before the addition of the drugs. The same amount of ^{14}C-TEA (0.15 µCi) was added to each test tube together with varying amounts of non-labelled TEA. Other drugs were always added to the media just before ^{14}C-TEA. At the end of the

incubation period the slices were separated from the media by filtration on cotton and weighed (wet weight).

Measurement of radioactivity

Samples of tissue and medium were prepared for radioactivity measurements in a Packard Tri-Carb liquid scintillation spectrometer (model 3375) as previously described (HOLM 1970a).

The results were expressed as the slice-to-medium (S/M) concentration ratio of ^{14}C, which was calculated as the counting rate per g slice (post-incubation wet weight)/counting rate per ml medium. The difference between the S/M ratio in the control experiment (without treatment) and experiment with treatment was expressed as per cent of the control value. The significance of these differences was estimated by "Student's t-test" (BRADFORD HILL 1966).

In some experiments the uptake of ^{14}C TEA by kidney slices (mmol per kg tissue) was calculated from the S/M ratio and the ^{14}C-TEA concentration of the medium (mmol per l medium).

Preparation of chromatograms.

Following incubation with ^{14}C TEA protein-free kidney extracts were prepared as described previously (HOLM 1970a) and chromatographed on paper by the method previously described (BRØEN CHRISTENSEN & HOLM 1969). Two different systems were used as the mobile phase: A. n-butanol:ethanol:glacial acetic acid:water (8:2:1:3) B. pyridine:n-butanol:water (3:2:3)

Results

Tetraethylammonium uptake as a function of the duration of incubation.

Fig. 1 shows the relationship between the uptake of TEA (2 µM) and the duration of incubation. The uptake was expressed as the slice-to-medium (S/M) concentration ratio. TEA is rapidly accumulated in an atmosphere of oxygen-carbon dioxide. The concentration of TEA in the tissue is thus already 5 times higher than that of the medium after incubation for 3 minutes, and a steady state distribution with S/M ratio values between 15 and 20 is obtained within ½ hr. In an atmosphere of nitrogen-carbon dioxide the S/M ratio reaches a constant value around 1 within 15 minutes.

Effect of metabolic inhibitors and low temperature on tetraethylammonium uptake.

Table 1 shows the effect of metabolic inhibitors and low temperature (19°) on the uptake of TEA (2 µM). The uptake was expressed as the slice-to-medium (S/M) concentration ratio. It is seen that the TEA uptake (30 minutes incubation) is reduced to one tenth of the control value in the presence of 500 µM 2,4-dinitrophenol and 1000 µM iodoacetate, whereas 10 µM ouabain has no effect on the uptake. Incubation at low temperature depresses the initial uptake (3 minutes incubation) by 40 per cent, but has no effect on the steady state S/M ratio (30 minutes incubation).

Effect of quaternary and tertiary amines on tetraethylammonium uptake.

Table 2 shows the effect of various quaternary and tertiary amines on the uptake of TEA (2 μM). The uptake was expressed as the slice-to-medium (S/M) concentration ratio after incubation for 30 minutes.

It is seen that choline carbacholine N[1]-methylnicotinamide and neostigmine depress the uptake of TEA. Furthermore the results show that the inhibitory action of the three former compounds is dose dependent, and that neostigmine is a more potent inhibitor of the uptake than any of the other three mono-quaternary ammonium compounds.

The two polymethylene bis-quaternary ammonium compounds decamethonium and hexamethonium did not inhibit the uptake to the same extent as the above-mentioned mono-quaternary ammonium compounds. Decamethonium was a more potent inhibitor of TEA uptake than hexamethonium, as the uptake was halved in the presence of 1 mM decamethonium, whereas hexamethonium at the same concentration had no effect on the uptake.

The quaternary ammonium compounds d-tubocurarine and hemicholinium 3 as well as the tertiary amines atropine and physostigmine seemed to be the most potent inhibitors of uptake

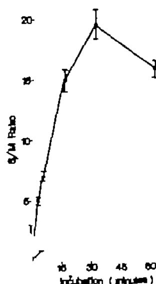


Fig. 1 Tetraethylammonium (TEA) uptake by mouse kidney slices as a function of the duration of incubation (minutes). The slice-to-medium (S/M) concentration ratio of TEA (2 μM) was measured in an atmosphere of oxygen-carbon dioxide (○) or nitrogen-carbon dioxide (●). Each circle represents the mean value of 6 experiments with S.E.M. (vertical bars for values higher than ± 0.1).

Table 1

Effect of metabolic inhibitors and low temperature (19°) on tetraethylammonium (TEA) uptake by mouse kidney slices. The slice-to-medium (S/M) concentration ratio of TEA (2 μM) was measured in an atmosphere of oxygen-carbon dioxide. Slices were incubated either in conjunction with treatment (in the presence of metabolic inhibitors or at low temperature) or without treatment (control). Results are given as the mean ± S.E.M. of values from 6 experiments. Figures in brackets indicate molar concentration of metabolic inhibitor

Treatment	Incubation (min.)	S/M ratio		Inhibition (%)
		Control	Treated	
2,4-Dinitrophenol (5×10^{-4} M)	30	14.2 ± 1.0	1.39 ± 0.03	90 ± 1)
Iodoacetate (10^{-3} M)	30	18.6 ± 0.7	2.1 ± 0.1	89 ± 1)
Ouabain (10^{-5} M)	30	19.6 ± 1.1	18.6 ± 0.9	6 ± 4
Low temp. 19	30	19.6 ± 1.2	19.5 ± 0.8	1 ± 6
Low temp. 19	3	5.0 ± 0.3	3.0 ± 0.2	38 ± 6**)

Mean percentage values differ significantly from zero: $P < 0.001$*), $P < 0.005$**).

Tetraethylammonium uptake as a function of the tetraethylammonium concentration in the medium.

Fig. 2 shows the relationship between the TEA uptake and the TEA concentration of the medium. In these experiments the uptake was expressed as mmol/kg tissue after incubation for ½ hr. The uptake was calculated from the S/M ratio and the concentration of TEA in the medium. The uptake was measured in an atmosphere of oxygen-carbon dioxide as well as in an atmosphere of nitrogen-carbon dioxide. Under anaerobic conditions the uptake is seen to be directly proportional to the TEA concentration of the medium (average slope or S/M ratio around 0.9), whereas this is not the case under aerobic conditions. The aerobic uptake thus seems to consist of at least two components: one that shows saturation with increasing TEA concentration in the medium, and one that increases proportionally with the external TEA concentration. The average slope (which is also the S/M ratio) of the latter component seems to be around 1.2.

The concentration ratio for the saturable component of the TEA uptake (Sa/M) can be calculated as the difference between the concentration ratio for the whole uptake and the concentration ratio for the linear component, namely 1.2. In fig. 3 the reciprocal values of Sa/M have been plotted against

the rat had a similar time course (McIsaac 1969) The uptake of TEA re quires energy as it is depressed in the presence of metabolic inhibitors (table 1) and also under anaerobic conditions (fig. 1). The results in table 1 also show that incubation at low temperature depresses the initial TEA uptake, but has no effect on the steady state distribution. The latter observation must mean that the influx and efflux at a steady state are equally reduced at a low temperature.

It has recently been shown that a large number of quaternary and tertiary amines inhibit decamethonium uptake by mouse kidney slices (Holm 1970a, b & c; Holm 1971a &b) Table 2 shows that the same agents – and in addition decamethonium – also inhibit TEA uptake. Our data do not allow any definite conclusions to be drawn concerning the nature of the inhibition, but it seems justifiable to assume that the above-mentioned inhibitors compete with TEA for a common carrier mechanism, which is capable of transporting quaternary ammonium compounds and tertiary amines in the cationic form. In this connection it is of particular interest that some of the compounds, which inhibit TEA uptake, are secreted by kidney tubules in the intact dog and hen. Choline is thus secreted in the dog and chicken (Vander 1962 Rennick 1958) N[1]-methylnicotinamide in the dog and chicken (Beyer et al. 1950 Sperber 1949) neostigmine in the hen (Roberts et al. 1965) and hexamethonium in the chicken (Rennick 1958)

As shown (fig. 2) the uptake of TEA under aerobic conditions can be divided into at least two components one that shows saturation and one that is proportional to the external TEA concentration. The saturable component is most likely to represent an accumulating carrier-mechanism for TEA. The linear component has a S/M ratio (or proportionality constant) of 1.2 and seems to be metabolically dependent, as it exceeds the anaerobic uptake. This part of the uptake therefore cannot merely represent a simple distribution of TEA in the extra and intracellular aqueous phase, but may be due to either an additional carrier mechanism to which TEA has an extremely low affinity (the concentration range studied is far below the saturation level) or a diffusion into the cells followed by an energy requiring binding to intracellular sites. It should be mentioned that the uptake of decamethonium by mouse kidney slices was also shown to consist of a saturable and a linear component (Holm 1970a & b)

It is concluded that the uptake of TEA by mouse kidney slices shows saturation, requires energy and is inhibited by structural analogues (quaternary and tertiary amines) These kinetics are similar to that described for the uptake of decamethonium by mouse kidney slices (Holm 1970a, b & c, Holm 1971a &b) which is consistent with the suggestion that TEA and decamethonium share a common specialized transport mechanism in mouse kidney slices (Holm 1971a) This transport mechanism *in vitro* is most likely to be

in part at least, identical with that responsible for the secretion of organic cations by the kidney

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the rat had a similar time course (McIsaac 1969) The uptake of TEA requires energy as it is depressed in the presence of metabolic inhibitors (table 1) and also under anaerobic conditions (fig. 1) The results in table 1 also show that incubation at low temperature depresses the initial TEA uptake, but has no effect on the steady state distribution. The latter observation must mean that the influx and efflux at a steady state are equally reduced at a low temperature.

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two different mechanisms, alter the target organ burden of mercury after methyl mercury exposure.

Retention of small amounts of mercury over a prolonged period of time is the principal mechanism underlying the hazards of methyl mercury Previous papers have discussed the biotransformation reaction only after a single injection of methyl mercuric chloride, and the results have been used to evaluate the pharmacokinetics of this compound. The role of the biotransformation reaction may not be the same with chronic administration of mercury as with a single injection. In the present study two different dose levels were tested since preliminary experiments indicated that the rate of biotransformation in the rat *in vivo* decreased with increasing doses.

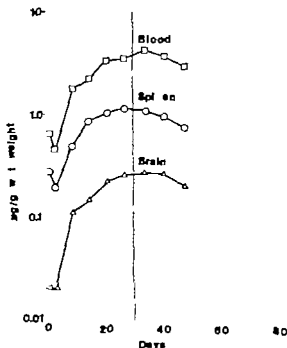


Fig. 1. Total mercury per g l blood, spleen and brain at different time intervals. The rats were injected every third day with 0.02 mg of mercury per rat as methyl mercuric cysteine. The last injection is indicated by the dotted line. Each rat received a total of 0.22 mg of mercury (1.1 mg Hg/kg) Each value represents the mean of organs from 3 rats (day 48 – 2 rats).

Materials and methods

Female rats weighing about 200 g were injected subcutaneously with a total of 1.1 mg/kg (dose A – 26 rats) or 32.5 mg/kg (dose B – 30 rats) of mercury given as methyl mercuric cysteine. Dose A was small (LD50 is 15–20 mg/kg, SWENSSON & ULFVARSON 1963) and approximately the same as that given previously in a single injection (NORSETH & CLARKSON 1970b). Dose B was chosen in order to give a brain concentration of mercury in the low range at which toxic signs might be expected (BERGLUND 1969). The total dose was given as 11 and 13 single doses, respectively at intervals of 3 days. The rats thus received 0.02 mg or 0.5 mg of mercury per injection, the highest total dose for the two groups being 0.22 mg and 6.5 mg after 30 or 36 days respectively. The molar ratio of cysteine to mercury in the injection solutions was 10. Cysteine was added because of severe skin necroses which occurred when the methyl mercuric chloride dissolved in Na_2CO_3 was injected, and because of the limited solubility of methyl mercuric chloride at physiological pH.

Groups of 3 rats were killed at intervals of 6 days. The first 2 groups given both dose levels respectively were killed on day 1 and 3, while the last group receiving dose B, was killed after an interval of 12 days.

The rats were sacrificed under slight ether narcosis, the blood collected from the abdominal aorta and the organs removed for preparation as described previously (Nor-

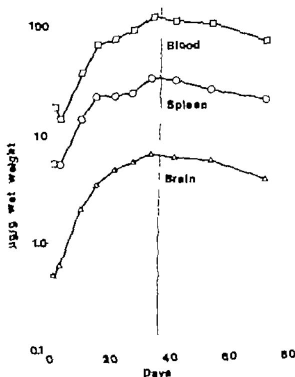


Fig. 2. Total mercury per g in blood, spleen and brain at different time intervals. The rats were injected every third day with 0.5 mg of mercury per rat as methyl mercuric cysteine. The last injection is indicated by the dotted line. Each rat received a total of 6.5 mg of mercury (32.5 mg Hg/kg). Each value represents mean of organs from 3 rats.

SETH & CLARKSON 1970b). The total mercury and inorganic mercury in the presence of methyl mercuric salts were determined as described in a previous paper (NORSETH & CLARKSON 1970a). Methyl mercuric chloride was prepared according to previously described methods and carefully controlled for contamination with inorganic mercury

Results

After repeated injections of dose A the mercury content in the blood increased to about 4 µg/ml after 30 days (fig. 1) At the time of the last injection of dose B the mercury content in the blood was about 120 µg/ml (fig. 2). There is thus no major difference between the ratio of the dose levels and the ratio of mercury content in the blood. Correspondingly the values 1.86 / and 1.80*/ of the dose found per ml blood for dose A and B respectively are not significantly different (table 1)

The mercury content in the spleen after injection of dose A was slightly more than 1 µg/g at the time of the last injection.

The corresponding value after dose B was slightly more than 30 µg/g (figs. 1 and 2). The same ratio as for the blood, between the two doses, was

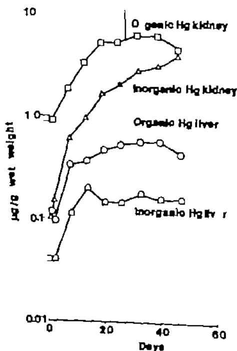


Fig. 3. Organic and inorganic mercury per g in liver and kidney at different time intervals for the same rats as shown in fig. 1.

Table 1

% of dose per g organ (ml).

No. of rats	Dose (mg/inj.)	Blood*	Kidney	Kidney org.	Liver	Brain	Spleen
9	0.02 (A)	1.86 ± 0.27	3.92 ± 0.52**	2.59 ± 0.37	0.33 ± 0.05	0.12 ± 0.02	0.56 ± 0.11
6	0.5 (B)	1.80 ± 0.24	1.44 ± 0.11	1.22 ± 0.11	0.32 ± 0.05	0.10 ± 0.01	0.52 ± 0.06

Values are given as mean ± S.D. Rats killed 3 days before and 1–5 days after the last injection are included in the table.
Total dose is corrected to the correct time period for all calculations.
* 6 rats for dose A and 5 rats for dose B.
** Significant differences between dose levels ($P < 0.001$).

also found for the spleen, and the relative amounts of the dose per g tissue were 0.56 / and 0.52 / respectively (table 1)

The brain contained 0.27 μg Hg/g after 30 days when dose A was injected. After dose B, the brain contained about 6.5 μg/g (figs. 1 and 2) Based on these values there seem to be a slightly higher accumulation of mercury relative to the dose for dose A, but the relative amounts of the dose per g brain, 0.12 / and 0 10*/ are not significantly different (table 1)

The blood, spleen and brain seemed to release mercury at approximately the same rate, but observations after the injection period are too few for any final conclusions. The relative amount of inorganic mercury was less than 5 / for blood, spleen and brain at all the time intervals tested.

The amount of total mercury in the liver when dose A was injected was slightly less than 1 μg/g after 30 days (fig. 3- The amount of total mercury in the liver after the injection of dose B was about 25 μg/g, giving the same ratio of about 30 for the two dose levels as that in the blood, spleen and brain (fig. 4) Correspondingly the values 0.33 / and 0.32 / are not significantly different (table 1). The relative amount of inorganic mercury in the

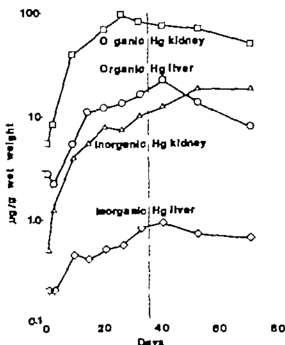


Fig. 4. Organic and inorganic mercury per g in liver and kidney for the same rats as shown in fig. 2.

liver varied from about 20% to 30% during the time period tested when dose A was injected. At the high dose level the relative amount of inorganic mercury never exceeded 10% most values being below 5% (calculated from figs. 3 and 4). There is thus a difference between the relative amounts of inorganic mercury in the liver at the two dose levels.

When dose A was injected, the kidney contained about 8 µg/g (fig. 3). For dose B, the corresponding value was 90 µg/g (fig. 4) The ratio between these two values indicates that a relatively higher amount of mercury was accumulated in the kidney with the lower dose. Dose A gave 3.92% of the total dose per gram of kidney at the time of the last injection, while only 1.44% was found with dose B (table 1). These values are clearly different.

Inorganic mercury in the kidney continued to increase after the end of the injection period (figs 3 and 4) The highest amount of inorganic mercury after the injection of dose A, 3.7 µg/g, was found after 48 days (last tested period). The relative amount of inorganic mercury in the kidney thus increased steadily from about 10% at day 1 to about 50% at day 48.

At the end of the experiment (72 days) inorganic mercury in the kidneys of rats given dose B was less than 20 µg Hg/g, which gives a relative amount of about 25% All single values for relative amount of inorganic mercury

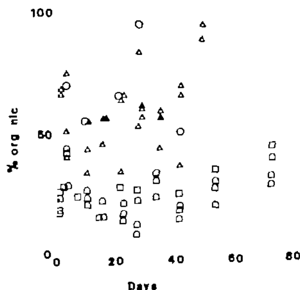


Fig. 5. Relative amount of inorganic mercury in single samples of faeces and caecal content at different time intervals. The symbols ○ and △ represent rats injected with 0.02 mg Hg/injection, faeces and caecal content, respectively The symbol □ represent faeces and □ caecal content from rats given 0.5 mg Hg/injection.

both in the liver and in the kidney were higher for dose A than for dose B.

When the relative amount of mercury in the kidney according to the dose was calculated for organic mercury only there was also a difference as shown by the values of 2.59% and 1.22% (table 1).

Inorganic mercury in the faeces and in caecal content from the rats receiving dose A was from 30% to 95% of the total amount of mercury in the samples analysed, most values being between 50% and 70% (fig. 5).

Faeces and caecal content collected from the rats receiving dose B contained a relative amount of inorganic mercury which varied from 10% to 40%, most values being between 15% and 30%. All single values except one after 3 days, were higher for dose A than for dose B. A total of 34 samples were analysed for dose A and 38 samples for dose B (fig. 5).

Discussion

There is a difference in the biotransformation rate of methyl mercury salts in the rat which is dose dependent. Based on this result it is possible to test the importance of this reaction in relation to the hazards of methyl mercury exposure. The target organ in methyl mercury poisoning is the brain, and inorganic mercury does not penetrate into the brain, as readily as the intact organo-mercurial (SWENSSON & ULFVARSON 1968). The brain is also the principal target organ for mercury vapour exposure, but even if mercury vapour in the blood is relatively rapidly oxydized to mercuric ions, it has been shown that most of the mercury penetrates into the brain as the unchanged vapour (MAGOS 1968). A much higher exposure to inorganic mercuric ion is probably necessary to produce signs of toxicity in the central nervous system. The target organ for poisoning with mercuric ions is the kidney. The kidney can, however tolerate much more mercury after chronic exposure than after acute exposure, i.e. showing no damage or functional disturbances. The importance of the kidney as target organ for inorganic mercury released from methyl mercuric salts *in vivo* is therefore difficult to predict. A possible combined effect with other kidney poisons such as cadmium, another important pollutant, must, however be borne in mind.

The present results support the assumption that the biotransformation reaction is of no significance in the development of the specific central nervous system signs in methyl mercury poisoning. A low degree of biotransformation with no indication of retention of inorganic mercury in the brain was reported in a previous paper (NORSETH & CLARKSON 1970b). The amount of inorganic mercury in the brain was also low in the present experiment. The differences in organ distribution between the rats exposed to the high and to the low dose were not responsible for any differences in the brain content of

mercury. The higher kidney content of mercury with the low dose might be expected to produce a lower brain level as compared to rats exposed to the high dose. No such differences were found. This is because the increased mercury content in the kidney represents only a very limited amount of the total body burden of mercury and is thus not reflected in the brain content. The higher kidney content of mercury cannot be explained exclusively by an increased biotransformation and accumulation of inorganic mercury by this organ. The excretion mechanism in the liver may be involved. There is a significantly lower amount of uncharged organo-mercurial in the kidney with the high dose. Selective uptake by the kidney of mercury after entero-hepatic circulation was postulated in a previous paper (NORSETH & CLARKSON 1971). If high doses of mercury inhibit the liver excretion mechanism, this would lead to a lower kidney content of mercury. The daily excretion of mercury in the bile after prolonged exposure to high doses has, however, not been tested.

Methyl mercury was injected as the cysteine complex in these experiments because of the low solubility of methyl mercuric chloride. The importance of the relative high doses of cysteine was tested in a preliminary experiment. The total mercury content in the kidney or the relative amount of inorganic mercury was the same after an intravenous injection of 1 mg Hg/kg of methyl mercuric cysteine and a corresponding injection of methyl mercuric chloride. Furthermore, the results reported by ULFVARSON (1962) are in good agreement with the present results. Calculating the relative amounts of the total dose in different organs from his results, the figures are within the range reported in the present paper for the brain, kidney and blood (table 1). The liver value reported by ULFVARSON is slightly higher. This may be related to the need of cysteine for the biliary excretion of methyl mercury as a small molecular weight compound (NORSETH & CLARKSON 1971), but is of no consequence for the target organ burden of mercury.

The relative amount of inorganic mercury in the faeces and in intestinal content was significantly lower with the high than with the low dose. The total body burden was not tested in these rats. Small differences in the excretion of mercury because of this difference, are therefore possible according to previous theories (NORSETH & CLARKSON 1971) which indicate a preferential excretion of inorganic mercury after methyl mercury exposure. BERGLUND (1969) found a relatively 20% higher body burden when rats were fed 5 mg Hg/kg as methyl mercuric hydroxide as compared to 0.2 mg Hg/kg. This result supports the assumption that biotransformation is important for the excretion, since both biotransformation and excretion are dose dependent. ULFVARSON (1969) could not, however, demonstrate a dose dependent rate of excretion after a single injection. A dose dependent biotransformation after a single injection was found in preliminary experiments.

Conflicting results have also been reported for a dose dependent rate of excretion in the mouse (ÖSTLUND 1969 ULFVARSON 1970· CLARKSON 1970). It is important to note, however that differences in the biotransformation rate are of no significance for the brain content of mercury in this experiment. The substrates for biotransformation in the intestines are probably methyl mercuric protein complexes (NORSETH & CLARKSON 1971) The results indicate that biotransformation takes place within the intestinal tract, the mercury compounds acting as substrates which would be excreted in any case. The mechanism of this reaction is still unknown, but is of considerable interest in relation to the treatment of methyl mercury exposure by unabsorbable resins and also in the mechanism of binding to these resins (CLARKSON *et al.* 1971)

Based on the previous demonstration of the biotransformation of methyl mercuric salts *in vivo* some doubt could be thrown on the validity of the one compartment model suggested for the excretion and retention of mercury after exposure to methyl mercury salts. Biotransformation resulting in time dependent distribution changes would indicate a model with more than one compartment to be more correct. The present results indicate, however that the deviations from the one compartment model caused by biotransformation are too small to alter the overall experimental fit to the proposed *theoretical* model (ULFVARSON 1962 BERGLUND & BERLIN 1969). The rat also seem to have a higher rate of biotransformation than the other animals tested, e.g. the mouse (NORSETH & CLARKSON 1970b· NORSETH 1971) The half-life of mercury in the mouse is shorter than in the rat, but other *interspecies* differences apart from the biotransformation rate such as biliary excretion or red cell to plasma ratio may explain these results (ULFVARSON 1962, 1969 & 1970· BERGLUND 1969 ÖSTLUND 1969· NORSETH & CLARKSON 1970a & b NORSETH 1970).

The distribution of mercury in the mouse is more like that in the human subject, thus supporting the usefulness of the one compartment model for the evaluation of human hazards of methyl mercury exposure, even if the model is not entirely correct, based on biochemical considerations.

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From the Laboratory of Teratology Karolinska Institutet,
Stockholm, Sweden

Studies on the Closure of the Ductus Arteriosus IX Transitory Effect of Phenoxybenzamine on Ductal Closure in the Guinea-Pig

By

P Y Hörnblad and K. B. Larsson

(Received September 28, 1971 Accepted October 28 1971)

Abstract: The effect of phenoxybenzamine on the closure of the ductus arteriosus in the guinea-pig was studied by means of the whole-body freezing technique. Foetuses were injected intraperitoneally with 1 mg phenoxybenzamine 10 minutes before surgical delivery and sacrificed 10 or 30 minutes later A delay in ductal closure was observed 10 minutes but not 30 minutes after delivery The observation suggests that ductal closure in the guinea-pig is not solely mediated through the α-adrenergic, cholinergic or histamine receptors.

Key-words: Ductus arteriosus – closure rate – whole-body freezing – phenoxybenzamine – guinea-pig.

By means of the whole-body freezing technique the ductus arteriosus can be fixed in a state which reflects its condition *in vivo* (Hörnblad & Larsson 1967a Hörnblad 1969a). This technique has been used for studies on ductal morphology mode of closure and closure rate as well as for studies of the mechanism of the ductal closure in a number of species (Hörnblad 1967 Hörnblad 1969b Hörnblad 1969c; Hörnblad & Friberg 1969 Hörnblad & Larsson 1967b Hörnblad *et al.* 1969). Moreover whole-body freezing was used for studies of pharmacological factors involved in oxygen-induced ductal closure (Hörnblad *et al.* 1970). Phenoxybenzamine (benzylytum NFN), which in the dose given induced a long-lasting blockage of cholinergic receptors, certainly also of α-adrenergic and of histamine receptors (Nickerson 1970), delayed ductal closure in the guinea-pig 10 minutes after birth. The specific action of phenoxybenzamine (POB) is not understood. Patent ductus arteriosus, one of the most common malformations in man, has never been produced in experimental animals. The present investigation was therefore designed to determine whether POB produced an unspecific reversible delay in ductal closure or a true patent ductus arteriosus.

Material and Methods

Full term multiparous guinea-pigs were brought to the laboratory from a commercial breeder at a time when symphysiolysis indicated the approach of spontaneous delivery (RECORD & McKEOWN 1955) Caesarean sections were performed under ether anaesthesia. The lower abdominal wall was injected with 5 ml of a 0.5 per cent solution of prilocaine chloride (citanest® AB Astra, Södertälje, Sweden) to keep the animals under slight general anaesthesia. After incision of the abdominal wall the lower part of the animal was immersed in saline at 38 and the uterus was gently extracted and kept below the surface of the saline. While still *in utero* each foetus received an intraperitoneal injection of 1 mg POB hydrochloride (dibenzyline® Smith Kline and French Laboratories Ltd., England). Ten minutes later the foetuses were delivered surgically and made to start breathing. The foetuses of each litter, after breathing for 10 and 30 minutes respectively were sacrificed and fixed in liquid nitrogen. After storage for a few days at −18 blocks were trimmed down to the mediastinum containing the ductus arteriosus, and were oriented for transverse sectioning. Cryostat sections 10 μ thick were made semi-serially throughout the ductus and one out of 15 sections was kept and stained with haematoxyline and eosin. In the section exhibiting the narrowest lumen of the ductus arteriosus, the inner diameter and the wall thickness of the ductus were measured in the narrowest point and in the perpendicular plane, as described previously (HÖRNBLAD & LARSSON 1967a). The mean inner diameter and wall thickness were calculated from the arithmetic means of these measurements. In this study 26 foetuses from eight litters were used.

Results

An open ductus was found in 10 of the 15 newborns after 10 minutes, while three were partially contracted and two were almost closed. In the group of 11 animals sacrificed at the age of 30 minutes however there was a minimal lumen in three newborns and no ductal lumen could be found in eight animals.

Discussion

In a previous study in POB treated guinea-pigs, the ductal closure rate was significantly reduced 10 minutes after delivery (HÖRNBLAD *et al.* 1970). This observation indicated that POB delayed ductal closure by blocking certain receptors normally involved in the closure mechanism. In the present investigation it was found that guinea-pigs, given POB intraperitoneally before surgical delivery exhibited a partially open ductus at the age of 10 minutes which corresponds to our previous study (HÖRNBLAD *et al.* 1970) but that 30 minutes after delivery the ductuses were closed.

POB causes a prolonged interference with the α-adrenergic, cholinergic and histamine receptors. The failure of POB to cause a patent ductus arteriosus after 30 minutes may indicate that these receptors may play a role but are not essential for the closure of the ductus.

This is not in agreement with observations in the lamb by BORN *et al.* (1956) They found a closure of the ductus during asphyxia and interpreted the closure as being due to circulating catecholamines. In this species cholinergic nerve fibers have recently been demonstrated in the ductus by electron microscopy (SILVA & IKEDA 1971).

Moreover there is less evidence of a direct relationship between the high amount of catecholamines demonstrated with the fluorescence method in human embryonic ductuses (ARONSON *et al.* 1970 BORÉUS *et al.* 1969) and the process of closure. The trigger factors involved in the closure of the ductus arteriosus are not yet understood. The fact that POB does not have a longlasting effect on ductal closure, indicates that α-adrenergic or cholinergic receptors are not the only factors involved in the closure mechanism

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From the Institute of Pharmacology University of Oslo, Blindern, Oslo 3, Norway

Para-Aminophenol Metabolism in an Established Cell Line with Liver-like Functions

By

E. Dybing and H. E. Rugstad

(Received October 7 1971 Accepted November 8, 1971)

Abstract. The clonal MH_1C_1-strain of rat hepatoma cells in culture metabolizes *p*-aminophenol (PAP), measured both by substrate disappearance and by PAP glucuronide formation. PAP-glucuronide was assayed by spectrophotometric determination of the diazotized product coupled with naphthyl-ethylenediamine. In addition to glucuronidation, some of the drug is metabolized apparently by other pathway(s). Cultured cloned rat fibroblasts also exhibit PAP-glucuronidation activity whereas they do not conjugate bilirubin. Chang cells do not glucuronidate PAP Flavaspidic acid has no effect on the PAP-glucuronidation in the liver cells nor in the fibroblasts, in contrast to the inhibitory action seen on bilirubin conjugation by the same liver cells.

Key-words: Drug metabolism – glucuronyl transferase – cell culture.

The glucuronyl conjugation of various endogenous and exogenous substrates is known to take place via the UDP-glucuronyl-transferase system in the endoplasmic reticulum of the liver mainly in the rough-surfaced microsomes (Fouts & Gram 1969) Cell cultures provide a method for the investigation of the direct action of drugs on cells, and also the metabolism of certain drugs in one particular type of cells, in the absence of the complex metabolic interactions which take place in the whole animal. Hitherto, such studies have been severely restricted because of the lack of established cell lines with functions like those of parenchymal cells.

A clonal strain of rat hepatoma cells (MH_1C_1) performs a series of liver specific functions after serial culture for years. These cells produce serum albumin (Richardson *et al.* 1969) tyrosine amino-transferase, and the ninth component of complement (Tashjian jr. *et al* 1970). They conjugate testosterone (Tashjian jr. *et al* 1970) with glucuronic acid, and produce coagulation factors (Rugstad & Prydz 1971) They have biochemical regulatory mechanisms intact (Tashjian jr. *et al* 1970 Rugstad & Prydz 1971). Rugstad *et al.* (1970) showed that these cells also conjugate bilirubin and ex—

crete conjugated bilirubin into the culture medium. These cells should therefore be a unique tool for studying the conjugation of other substrates, i.e. drugs. Furthermore, this system might prove useful in studying the effect of a single chemical stimulus in the drug conjugation process. Lastly a comparison of results obtained by using these cultured liver-like cells with those from experiments with other cell lines, might give evidence of cell specifity for drug conjugation. *p*-Aminophenol (PAP) the hydroxylated metabolite of aniline, was chosen as substrate in this study

Materials and Methods

Para-aminophenol (PAP) was obtained from Norsk Medisinaldepot (NMD); β-glucuronidase (Type I bacterial) N-1-naphtyl-ethylenediamine diHCl (NED) from the Sigma Company Glucaro-1 4-lactone was purchased from Calbiochem. Flavaspidic acid-N-glucaminate was a gift from Dr Esa Aho, Laaketehdas Leiras, Turku, Finland.

Drug assays. The rate of PAP disappearance was determined using the method of KATO & GILLETTE (1965).

PAP-glucuronide formation was estimated by a method similar to that used by LEVVY & STOREY (1949) for o-aminophenyl-glucuronide. The glucuronide is diazotized and coupled with NED in a 2 M phosphate buffer at pH 2.5 The coloured product is stable and has a maximum absorbance at 560 nm in a Beckmann B-spectrophotometer

Method of cell culture. The cells were grown in Dulbecco's modified Eagle medium and (unless otherwise stated) supplemented with 2.5 % foetal calf serum and 15 % horse serum. Penicillin 100 U/ml, streptomycin 0.1 g/ml, and nystatin 60 U/ml were added.

A clonal strain derived from Morris rat hepatoma no. 7795 designated MH_1C_1, was used. The cells were grown in Carrell flasks with a diameter of 8 cm, containing 10 ml of medium. Subcultures (1:2 to 1:4 split) were made after incubation with 0.25 % trypsin in a buffered 0.02 % EDTA-solution. Full-grown or nearly full-grown cultures were used (protein content 3–5 mg per flask), usually 6–8 days after subculture. Six to ten identical subcultures were made for each set of experiments.

In addition to the MH_1C_1-cells, some experiments were performed with a clonal strain of rat fibroblasts (R5) derived from a rat thyroid gland, or the Chang liver cell line (CHANG 1954)

Cell protein was determined according to OYAMA & EAGLE (1956) using bovine serum albumin as standard.

Experimental procedure. Cell cultures were incubated with substrate at various concentrations in a final volume of 10 ml at 37° in a closed system after pH-correction with CO_2 to pH 7.4 as indicated by the phenol red colour in the medium. To prevent auto-oxidation L-ascorbic acid was added to PAP stock solutions to make a final concentration of L-ascorbic acid of 1.0 mM in the incubation medium. Aliquots of 0.25 – 1.0 ml were withdrawn at desired time intervals and the PAP-disappearance was checked immediately after the end of the experiments. Samples for PAP-glucuronide determination could be frozen for later assay without loss of activity

Separate control experiments were done to ensure that the coloured product in this assay was derived from the PAP-glucuronide. Samples containing the assumed glucuronide were incubated for 1 hour at 37 with 2500 U β-glucuronidase in a 0.1 M phosphate buffer pH 7.0, with β-glucuronidase and varying concentrations of glucarolactone (specific β-glucuronidase inhibitor), or with buffer only

Flavaspidic acid (FA), an inhibitor of bilirubin glucuronidation, was incubated at concentrations of 0.16 and 0.08 mM together with 0.25 mM PAP as substrate.

Results

PAP assays

The compound PAP is unstable in aqueous solutions. For 5 to 6 hours at 37° in the dark, no changes in flasks incubated with PAP without cells were seen, but most of the substrate disappeared after 24 hours, presumably due to auto-oxidation, even when anti-oxidants were used. Therefore estimation of PAP-disappearance (Kato & Gillette 1965) was carried out immediately after the end of each experiment. However once the glucuronide was formed, this compound was stable and could be frozen for later assay

The method used for PAP-glucuronide determination was checked by the use of β-glucuronidase and glucarolactone. Medium containing 0.25 mM was incubated with MH_1C_1 cells for 4 hours. Almost 90 / of the substrate lost (Kato & Gillette 1965) could be recovered after incubation with 2500 U β-glucuronidase per ml reaction mixture for one hour This recovery could be completely prevented by 10 mM glucarolactone. When the diazota

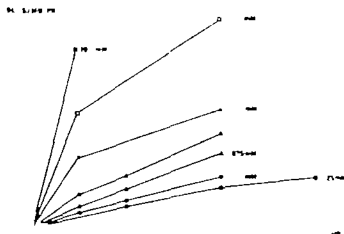


Fig 1. Disappearance of PAP in culture medium of rat hepatoma cells (MH_1C_1) t different substrate concentrations. (Ordinate: Disappearance of PAP expressed as µmol per mg cell protein. Abscissa: Time in hours.)

tion procedure was used, no coloured product was formed when the test samples were pretreated with the same amount of β-glucuronidase. The addition of glucarolactone to the β-glucuronidase gave values identical to those obtained from untreated test samples. Correlating these results, a spectrophotometric extinction coefficient for PAP-glucuronide was estimated (39 14 mM⁻¹ cm⁻¹)

PAP-disappearance in MH_1C_1-cultures

Using the MH_1C_1 cell line, the rate of PAP-disappearance was related to substrate concentration and time from fig. 1 it can be seen that this process is fairly linear at concentrations between 0.25 mM and 0.75 mM from 0 to 5 hours. The linearity is lost at the lowest concentration on incubation for more than this time interval. At concentrations from 1 0 mM and higher the linearity also disappears with shorter times of incubation. Experiments with higher concentrations of substrate could not be performed because of the instability and insolubility of PAP Samples in flasks without cells did not show any loss of substrate.

PAP-glucuronide formation in MH_1C_1 cultures

When measuring the rate of appearance of the metabolite PAP-glucuronide at varying substrate concentrations, a somewhat different picture is obtained

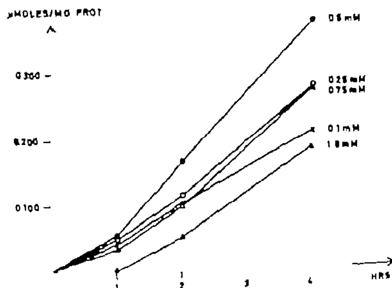


Fig. 2. Appearance of PAP-glucuronide in culture medium of rat hepatoma cells (MH_1C_1) at different substrate concentrations. Ordinate: Appearance of PAP-glucuronide expressed as µmol per mg cell protein. Abscissa: Time in hours

Table 1

MH_1C_1 cells were incubated with 2.5 μmol of PAP for 4 hours at 37° in a medium containing serum or without serum respectively. Total PAP disappearance and PAP glucuronide formation were determined as described. Mean ± S.D. Numbers of flasks in brackets.

Incubation medium	PAP disappearance μmol per mg cell protein per hour	PAP-glucuronide formation μmol per mg cell protein per hour
With serum	55.8 ± 5.0 (4)	49.5 ± 3.0 (4)
Without serum	55.2 ± 3.0 (4)	50.1 ± 5.0 (4)

(fig. 2). There is a reaction lag during the first hour of incubation, so that linearity is not reached. This lag increases with increasing amounts of substrate. Furthermore, the highest rate of PAP-glucuronide formation is reached at a substrate concentration of 0.5 mM. At higher concentrations the amount of glucuronide formed decreases. Incubation of substrate without cells did not reveal any glucuronide.

Incubating the cells with 0.25 mM PAP in a medium containing serum did not result in significant differences as compared with substrate disappearance and glucuronide formation when the experiments were performed in a serum-free medium (table 1).

Table 2

PAP disappearance and PAP-glucuronide formation in medium containing 2.5 μmol PAP incubated with different cell strains for 4 hours at 37°. Mean ± S.D. Numbers of flasks in brackets.

Cell Type	PAP disappearance μmol per mg cell protein and hour	PAP-glucuronide formation μmol per mg cell protein and hour	Glucuronide in % of total disappearance
MH_1C_1 (clonal rat hepatoma)	61.3 ± 6.1 (5)	52.8 ± 6.1 (5)	87.7
R 5 (clonal rat fibroblast)	45.0 ± 3.3 (4)	18.0 ± 2.8 (4)	40.0
Chang (derived human liver)	25.8 ± 4.8 (4)	0 (4)	

Table 3

Effect of flavaspidic acid (FA) on PAP-glucuronide appearance in culture medium containing 2.5 µmol PAP after incubation for 4 hours with rat hepatoma cells (MH_1C_1) and rat fibroblasts (R5). Amount of PAP-glucuronide given as nmoles per mg cell protein per hour. Numbers are mean of duplicate flasks.

Cell Type	PAP alone	PAP + 0.16 mM FA	PAP + 0.08 mM FA
MH_1C_1 (clonal rat hepatoma)	60.0	61.3	65.0
R 5 (clonal rat fibroblast)	19.0	20.8	20.3

PAP-metabolism in other cell lines

Both PAP-disappearance and PAP-glucuronidation were evident in fibroblast cultures (table 2) however the PAP-disappearance rate and relative amount of glucuronide formed were lower than in the MH_1C_1-cell line. At a substrate concentration of 0.25 mM PAP 40 / of the amount lost appeared as its glucuronide, verified by using β-glucuronidase and glucarolactone as described. The Chang cells did not produce any PAP-glucuronide at all although some of the substrate disappeared (table 2).

Effect of flavaspidic acid.

Experiments with simultaneous incubation of PAP together with flavaspidic acid in MH_1C_1 and fibroblast cultures did not reveal any inhibition of the glucuronide formation (table 3). Flavaspidic acid influenced the phenol-PAP-assay but when correcting for this (also standards for each of the FA-concentrations) no effect on the amount of PAP which disappeared could be seen when using MH_1C_1 cells.

Discussion

The method used for PAP-glucuronide assay is simple and reproducible. That the coupling assay with NED is actually a measure of PAP-glucuronide was verified using β-glucuronidase and glucarolactone, a specific β-glucuronidase inhibitor.

The appearance of PAP-glucuronide in the medium is maximal at a substrate concentration of 0.5 mM. Increasing substrate concentration leads to diminishing amounts of glucuronide in the medium. The overall glucuronidation in cell cultures is a complex process involving several steps (i. e. uptake of substrate, intracellular transport, enzymatic coupling, and finally

transport and egress of the glucuronide). Increasing amounts of PAP might inhibit any of these processes, and elucidation of this problem requires further investigation. The overall disappearance rate of PAP however increases with increasing substrate concentration, indicating that other path ways of drug metabolism with different substrate optima may take place.

The ability of fibroblast-derived cells to metabolize PAP is interesting, and it is in line with the findings that considerable drug metabolism can take place in tissues other than the liver An inducible glucuronyltransferase activity using *o*-aminophenol as substrate has been found in mouse and guinea pig skin (DUTTON & STEVENSON 1962) The fibroblasts used in the present experiments, however do not conjugate bilirubin (RUGSTAD *et al.* 1970)

No glucuronidation of PAP occurred in the Chang cells neither do these cells conjugate bilirubin (BRATTLID, personal communication)

Flavaspidic acid had no effect on the PAP-metabolism either in MH_1C_1 cells or in fibroblasts, whereas it has an inhibitory action on bilirubin con jugation both *in vivo* and by MH_1C_1 cells (RUGSTAD *et al* 1970)

PAP-glucuronidation occurs at the same rate when no serum is present in the incubation medium, indicating that there is no significant protein binding. The glucuronidation of bilirubin by MH_1C_1-cells, however, is negligible when the albumin concentration is zero (BRATTLID & RUGSTAD, unpublished results).

The overall glucuronidation process of PAP thus shows differences as compared to that of bilirubin, both in respect to cell line specificity and action of an inhibitor This could mean that there are more than one glucuronyl transferase operating when substrates are coupled with glu curonate. We believe that the investigation of conjugation reactions of various other drugs using the MH_1C_1-cells and fibroblasts might prove of value in assessing drug action.

Acknowledgements

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From the Department of Pharmacology University of Copenhagen,
20 Juliane Maries Vej, DK-2100 Copenhagen Ø, Denmark

In Vitro Studies on the Uptake of ^{14}C Labelled Carbamoylcholine in Mouse Kidney*)

By

Jan Holm

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Abstract. Carbamoylcholine uptake by mouse kidney slices was studied. The slices were incubated in Krebs-Ringer bicarbonate medium (37° pH 7.4) containing ^{14}C-carbamoylcholine, and the ^{14}C-slice-to-medium (S/M) concentration ratio was measured. Carbamoylcholine (2 µM) was rapidly accumulated in oxygen-carbon dioxide (95:5) atmosphere, and a steady state with S/M ratio around 10 was obtained within ½ hr The radioactive material extracted corresponded chromatographically to authentic carbamoylcholine. The S/M ratio was significantly reduced in the presence of metabolic inhibitors or various quaternary and tertiary amines. In nitrogen-carbon dioxide (95:5) atmosphere the S/M ratio (2 µM) reached a constant value around 1 within ½ hr Incubation at 19° markedly depressed the 3 min. S/M ratio, but increased the steady state S/M ratio (1 hr). Under anaerobic conditions carbamoylcholine uptake (mmol/kg tissue; 1 hr incubation) was directly proportional to the external carbamoylcholine concentration. The aerobic uptake consisted of two components: a saturable one (maximum capacity 7 mM/kg; half saturation concentration 700 µM) and another one that increased proportionally with the external concentration. It is concluded that carbamoylcholine is taken up against a concentration gradient by a saturable, energy dependent process which is inhibited by structural analogues.

Key words: Carbamoylcholine – transport – mouse kidney slices – *in vitro.*

In association with studies on the renal transport of quaternary ammonium compounds we have shown that mouse kidney slices accumulate decamethonium by a saturable, energy dependent process (HOLM 1970a) Recent investigations suggested that in mouse kidney slices, decamethonium and carbamoylcholine share a common transport mechanism (HOLM 1970c).

We therefore found it of interest to study the uptake process of carbamoylcholine in the mouse kidney for the purpose of comparing it with that

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*) A preliminary report on this study was presented at the XXIV Scandinavian Pharmacological Meeting, Oslo 1970.

of decamethonium. The present experiments were performed in order to establish whether the uptake of ^{14}C-carbamoylcholine by mouse kidney slices follows saturation kinetics, requires energy and is inhibited by structural analogues (compounds possessing positively charged nitrogen atoms at physiological pH). The possibility of a metabolic transformation of ^{14}C-carbamoylcholine was investigated by paper chromatography (method BRØDSGAARD CHRISTENSEN & HOLM 1969).

Materials and methods

^{14}C-methyl-carbamoylcholine chloride (specific activity 10.4 mci/mM) was supplied by the Radiochemical Centre, Amersham, England.

The following non-labelled compounds were used: carbamoylcholine chloride (Ph. Nord. 1963), neostigmine bromide (Ph.Nord. 1963), physostigmine salicylate (Ph.Nord. 1963), atropine sulfate (Ph.Nord. 1963), d-tubocurarine dichloride (Abbott), hemicholinium-3 (Aldrich, Chemical Co. Inc., U.S.A.), choline chloride (Merck), hexamethonium dibromide (May & Baker Ltd.), decamethonium dibromide (syncurine® Burroughs Wellcome & Co.), decamethonium diiodide (Burroughs Wellcome & Co.), ouabain (g-strophantin, Ph.Nord. 1963), 2,4 (α) dinitrophenol (Merck) and iodoacetic acid (Merck).

Experimental procedure.

Slices of mouse kidneys were prepared and used as previously described (HOLM 1970a). Male albino mice of a single strain (NMRI) with a body weight of 28–32 g were decapitated and bled. Four slices were cut from each kidney with a razor blade. Each of the experiments recorded in table 1 and 2 was carried out as a paired comparison with kidney tissue from two animals. Eight slices (total wet weight: 150–200 mg) from two kidneys (one kidney from each animal) were placed in a test tube, which contained 20 ml Krebs-Ringer bicarbonate solution with glucose (11 mM/l). This procedure made it possible to incubate kidney tissue from each animal (distributed in two test tubes) simultaneously under different experimental conditions (with or without treatment). Each animal thus served as its own control: method of paired comparison (BRADFORD HILL 1966). In the remainder of the experiments each test tube contained only kidney slices from the same animal (both kidneys).

Incubations were carried out by shaking (60 oscillations/min.) the test tubes at 37° (pH 7.4) and bubbling a 5:95 v/v % mixture of either carbon dioxide:oxygen or carbon dioxide:nitrogen through the medium. The slices were submerged in the salt solution for 10 minutes before the addition of the drugs. The same amount of ^{14}C-carbamoylcholine (0.4 µci) was added to each test tube together with varying amounts of non-labelled carbamoylcholine. Other drugs were always added to the media just before ^{14}C-carbamoylcholine. At the end of the incubation period the slices were separated from the media by filtration on cotton and then weighed (wet weight).

Measurement of radioactivity

Samples of tissue and medium were prepared for radioactivity measurements in a Packard Tri-Carb liquid scintillation spectrometer (model 3375 and 314 EX) as previously described (HOLM 1970a).

The results were expressed as the slice-to-medium (S/M) concentration ratio of ^{14}C, which was calculated as the counting rate per g slice (post-incubation wet weight)/counting rate per ml medium. The difference between the S/M ratio in the control (*without treatment*) *and experimental groups with treatment was expressed as per* cent of the control value. The significance of these differences was estimated by Student's t-test (BRADFORD HILL 1966).

In some experiments the uptake of ^{14}C-carbamoylcholine by kidney slices (mmol per kg tissue) was calculated from the S/M ratio and the ^{14}C-carbamoylcholine concentration of the medium (mmol per l medium).

Preparation of chromatograms.

Following incubation with ^{14}C-carbamoylcholine, protein-free kidney extracts were prepared as described (HOLM 1970a) and chromatographed on paper by the method previously described (BROBY CHRISTENSEN & HOLM 1969). Two different systems were used as the mobile phase: A, n-butanol:ethanol:glacial acetic acid:water (8:2:1:3). B, pyridine:n-butanol:water (3:2:3).

Results

Carbamoylcholine uptake as a function of the duration of incubation.

Fig. 1 shows the relationship between the uptake of carbamoylcholine (2μM) and the duration of incubation. The uptake was expressed as the slice-to-medium (S/M) concentration ratio Carbamoylcholine is seen to be rapidly accumulated in an atmosphere of oxygen-carbon dioxide. The concentration of carbamoylcholine in the tissue is thus already more than 3

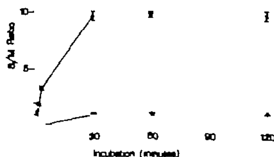


Fig. 1 Carbamoylcholine uptake by mouse kidney slices as a function of the duration of incubation (minutes). The slice-to-medium (S/M) concentration ratio of carbamoylcholine (2μM) was measured in an atmospere of oxygen-carbon dioxide (○) or nitrogen-carbon dioxide (●). Each circle represents the mean value of 6 experiments with S.E.M. (vertical bars).

times higher than that of the medium after incubation for 3 minutes, and a steady state distribution with S/M ratio values around 10 is obtained within ½ hr In an atmosphere of nitrogen-carbon dioxide the S/M ratio reaches a constant value around 1 within ½ hr

Effect of metabolic inhibitors and low temperature on carbamoylcholine uptake.

Table 1 shows the effect of metabolic inhibitors and low temperature (19°) on the uptake of carbamoylcholine (2μM). The uptake was expressed as the slice to-medium (S/M) concentration ratio. It is seen that the carbamoylcholine uptake (60 minutes incubation) is reduced to one tenth of the control value in the presence of 500 μM 2,4-dinitrophenol and 1000 μM iodoacetate, while 10 μM ouabain has only a slight but statistically significant effect on the uptake. Incubation at low temperature halves the initial uptake (3 minutes incubation) but increases the steady state S/M ratio (60 minutes incubation) by almost one half.

Table 1

Effect of metabolic inhibitors and low temperature (19°) on carbamoylcholine uptake by mouse kidney slices. The slice-to-medium (S/M) concentration ratio of carbamoylcholine (2 μM) was measured in an atmosphere of oxygen-carbon dioxide. Slices were incubated either with treatment (in the presence of metabolic inhibitors or at low temperature) or without (control). Results are given as the mean ± S.E.M. of values from 6 experiments. Figures in brackets indicate molar concentration of the metabolic inhibitor.

Treatment	Incubation (min.)	S/M ratio Control	Treated	Difference (%)
2,4-Dinitrophenol (5×10^{-4} M)	60	9.7 ± 0.2	0.99 ± 0.02	− 89 ± 1
Iodoacetate (10^{-3} M)	60	11.1 ± 0.8	1.19 ± 0.02	− 89 ± 1
Ouabain (10^{-5} M)	60	10.0 ± 0.8	9.3 ± 0.4	− 6.8 ± 1.7*
Low temperature 19	60	9.2 ± 0.5	13.2 ± 0.9	+ 45 ± 12 **
Low temperature 19	3	4.1 ± 0.2	2.1 ± 0.1	− 49 ± 3

Mean percentage values differ significantly from zero $P < 0.001$ $P < 0.01^{*}$ $P < 0.02^{**}$

Table 2

Effect of various drugs (quaternary and tertiary amines) on carbamoylcholine uptake by mouse kidney slices. The slice-to-medium (S/M) concentration ratio of carbamoylcholine (2 µM) was measured in an atmosphere of oxygen-carbon dioxide. Slices were incubated (1 hr) either with carbamoylcholine only (control) or with carbamoylcholine plus drug (treated). Results are given as mean values ± S.E.M. Figures in brackets indicate molar concentration of drug added.

Treatment	No. of exp.	S/M ratio Control	Treated	Difference (%)
Choline (10^{-4})	6	12.2 ± 0.6	11.9 ± 0.9	− 3 ± 4
Choline (10^{-3})	6	10.6 ± 0.4	5.4 ± 0.1	−49 ± 1
Neostigmine (10^{-4})	7	12.6 ± 0.8	4.5 ± 0.1	−64 ± 2
Neostigmine (10^{-3})	7	10.9 ± 0.8	1.70 ± 0.03	−84 ± 2
Decamethonium (2×10^{-5})	6	11.6 ± 0.6	11.3 ± 0.8	− 2 ± 5
Decamethonium (64×10^{-5})	6	11.5 ± 0.5	7.6 ± 0.3	−34 ± 3
Hexamethonium (10^{-4})	6	11.2 ± 0.6	8.5 ± 0.5	−23 ± 7 **
Hexamethonium (10^{-3})	6	8.7 ± 0.6	4.7 ± 0.3	−45 ± 3
d-Tubocurarine (22×10^{-4})	6	9.9 ± 0.9	4.4 ± 0.3	−54 ± 4
Hemicholinium −3 (10^{-3})	6	13.0 ± 0.7	1.39 ± 0.04	−89.2 ± 0.5
Physostigmine (22×10^{-5})	5	11.4 ± 0.6	6.0 ± 0.4	−47 ± 2
Physostigmine (22×10^{-4})	6	13.2 ± 0.8	3.9 ± 0.4	−71 ± 1
Atropine (2×10^{-3})	6	1..2 ± 0.3	3.4 ± 0.1	72 ± 1

$P < 0.001$
$P < 0.05$

Effect of quaternary and tertiary amines on carbamoylcholine uptake

Table 2 shows the effect of various quaternary and tertiary amines on the uptake of carbamoylcholine (2µM). The uptake was expressed as the slice-to-medium (S/M) concentration ratio after incubation for 60 minutes.

It is seen that the mono-quaternary ammonium compounds choline and neostigmine depress the uptake of carbamoylcholine. Neostigmine which inhibited the uptake in a dose-dependent manner was a more potent inhibitor than choline, as the uptake was reduced by two thirds in the presence of 100 µM neostigmine, whereas choline at the same concentration had no effect on the uptake.

The two polymethylene bis-quaternary ammonium compounds decamethonium and hexamethonium did not inhibit the uptake to the same extent as neostigmine. Hexamethonium, which inhibited carbamoylcholine uptake in a dose-dependent manner was a weaker inhibitor than decamethonium,

as the uptake was reduced by one third in the presence of 640 μM decamethonium, but only by one fourth in the presence of 1 mM hexamethonium.

Furthermore, it is seen that the quaternary ammonium compounds d-tubocurarine and hemicholinium-3 were more potent inhibitors of carbamoylcholine uptake than any of the other quaternary ammonium compounds mentioned above.

The tertiary amines atropine and physostigmine depressed the uptake of carbamoylcholine. Physostigmine, which inhibited the uptake in a dose-dependent way was a weaker inhibitor than atropine, as the uptake was reduced by three fourths in the presence of 20 μM atropine, but only halved by 22 μM physostigmine. Furthermore, the results show that atropine is a more potent inhibitor of the uptake than any of the other quaternary and tertiary amines in table 2.

Carbamoylcholine uptake as a function of the carbamoylcholine concentration in the medium.

Fig. 2 shows the relationship between the carbamoylcholine uptake and the carbamoylcholine concentration of the medium. In these experiments the uptake was expressed as mmol/kg tissue after incubation for 1 hr. The up-

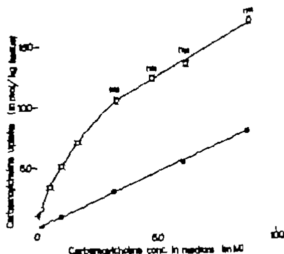


Fig. 2. Carbamoylcholine uptake by mouse kidney slices as a function of the carbamoylcholine concentration in the medium. The carbamoylcholine uptake (mmol/kg tissue; 1 hr incubation) was measured in an atmosphere of oxygen-carbon dioxide (○) or nitrogen-carbon dioxide (●). The circles represent mean values with S.E.M. (vertical bars for values higher than ± 0.1 mM), and figures in brackets indicate number of experiments. Unless otherwise stated, the number of experiments was 6.

take was calculated from the *S/M ratio and the concentration of carbamoylcholine* in the medium. The uptake was measured in an atmosphere of oxygen-carbon dioxide as well as in an atmosphere of nitrogen-carbon dioxide. Under anaerobic conditions the uptake is seen to be directly proportional to the carbamoylcholine concentration of the medium (average slope or S/M ratio around 0.9), whereas this is not the case under aerobic conditions. The aerobic uptake thus seems to consist of at least two components one that shows saturation with increasing carbamoylcholine concentration in the medium, and another that increases proportionally with the external carbamoyl choline concentration. The average slope (which is also the S/M ratio) of the latter component seems to be around 1.2.

The concentration ratio for the saturable component of the carbamoyl choline uptake (S_s/M) can be calculated as the difference between the concentration *ratio for the whole uptake and the concentration ratio for the* linear component, namely 1.2. In fig. 3 the reciprocal values of S_s/M have been plotted against the carbamoylcholine concentration of the medium according to the method of DIXON & WEBB (1959) for the quantitative evaluation of enzymatic reactions. As the relation appears to be linear it is possible to calculate the maximum capacity of this uptake process as the reciprocal value of the slope of the line and to read the half saturation concentration as the negative value of the point at which the line intersects the abscissa. Thus calculated the maximum capacity is found to be 7 mM kg^{-1} *and the half saturation* concentration, 700 μM.

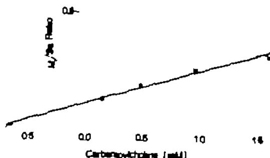


Fig. 3. Relationship between the reciprocal values of the concentration ratio for the saturable carbamoylcholine uptake (M/S_s) and the external carbamoylcholine concentration. The concentration ratio for the saturable carbamoylcholine uptake (S_s/M) was calculated as the difference between the concentration ratio for the whole uptake and the concentration ratio for the linear component (1.2): $S_s/M = S/M - 1.2$.

Paper chromatographic results

Protein-free extracts from kidney slices, which had been incubated (1 hr) in an atmosphere of oxygen-carbon dioxide with ^{14}C-carbamoylcholine (2 μM) were subjected to paper chromatography using the two systems described in the method section. The radioactivity of the chromatograms could not be distinguished from that of authentic ^{14}C-carbamoylcholine.

Discussion

The present results show that in slices of mouse kidney carbamoylcholine is concentrated as the unchanged compound. A rapid accumulation of carbamoylcholine occurs under aerobic conditions (fig. 1) since a steady state distribution is already obtained within ½ hr incubation. The uptake of carbamoylcholine requires energy as it is depressed in the presence of metabolic inhibitors (table 1) and under anaerobic conditions (fig. 1). The results in table 1 also show that incubation at low temperature depresses the initial carbamoylcholine uptake, but increases the steady state distribution ratio. The latter observation suggests – since influx equals efflux at a steady state – that the efflux is more reduced than the influx at a low temperature.

It has recently been shown that a large number of quaternary and tertiary amines inhibit decamethonium uptake by mouse kidney slices (HOLM 1970a, b & c, HOLM 1971) Table 2 shows that the same agents – and in addition decamethonium – also inhibit carbamoylcholine uptake. Our data do not allow of any definite conclusions concerning the nature of the inhibition, but it seems justifiable to assume that the above-mentioned inhibitors compete with carbamoylcholine for a common carrier mechanism, which is capable of transporting quaternary ammonium compounds and tertiary amines in the cationic form. In this connection it is of particular interest that some of the compounds, which inhibit carbamoylcholine uptake, are secreted by kidney tubules in the intact dog and hen. By this mechanism choline is secreted in the dog and chicken (VANDER 1962 RENNICK 1958), neostigmine in the hen (ROBERTS *et al.* 1965) and hexamethonium in the chicken (RENNICK 1958)

As shown (fig. 2) the uptake of carbamoylcholine can under aerobic conditions be divided into at least two components one that shows saturation and one that is proportional to the external carbamoylcholine concentration. The saturable component most likely represents the accumulating carrier mechanism for carbamoylcholine. It should be mentioned that the uptake of decamethonium by mouse kidney slices was also shown to consist of a saturable and a linear component (HOLM 1970a & b).

It is concluded that the uptake of carbamoylcholine by mouse kidney

slices shows saturation, requires energy and is inhibited by structure analogues (quaternary and tertiary amines). These kinetics are similar to that described for the uptake of decamethonium by mouse kidney slices (HOLM 1970a, b & c; HOLM 1971) which is consistent with the suggestion that carbamoylcholine and decamethonium *share a common specialized transport* mechanism in mouse kidney slices (HOLM 1970c). This transport mechanism *in vitro* is most likely to be in part at least identical with that responsible for the secretion of organic cations by the kidney.

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From the Institute of Medical Physiology C, University of Copenhagen,
71, Rådmandsgade, DK 2200 Copenhagen, Denmark

Effect of a Single, Large Intramuscular Dose of Bendroflumethiazide (Centyl®) on Calcium Metabolism in Rats

By

F Schønau Jørgensen

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Abstract A single, large dose of bendroflumethiazide was given intramuscularly in rats to investigate a possible extrarenal effect on calcium metabolism. Neither in normal, parathyroidectomized, thyroidectomized or thyro-parathyroidectomized rats nor by concomitant nephrectomy or parathyroid hormone administration, could any effect of bendroflumethiazide on the serum calcium concentration be demonstrated. The present findings are discussed in relation to previous experiments done in dogs and human subjects which suggested either a stimulating effect on the parathyroid glands or a potentiating effect of the thiazides on the action of the parathyroid hormone.

Key-words: Bendroflumethiazide – serum calcium concentration nephrectomy

The thiazide diuretics produce a decrease in renal calcium excretion (Lamberg & Kuhlbäck 1959 Lichtwitz *et al.* 1961 Seitz & Jaworski 1964 Higgins *et al.* 1964 Thomas *et al* 1965 Nassim & Higgins 1965 Duarte & Bland 1965 Yendt *et al* 1966 Harrison & Rose 1968 Parfitt 1969). In rats this effect is independent of the calcium regulating hormones from the thyroid and the parathyroid glands although a change in the concentration ratio of these two hormones in the blood during thiazide administration seems to occur (Jørgensen 1971b)

Pickleman *et al* (1969) found hypercalcaemia in dogs and hyperplasia of the parathyroid glands after long-term thiazide administration suggesting a stimulating effect of the thiazides on the parathyroid gland. Investigations in human subjects points to an extrarenal effect of the thiazides on calcium metabolism. Seitz & Jaworski (1964) Yendt *et al* (1966) and Harrison & Rose (1968) found an initial hypercalcaemia during thiazide administration. Parfitt (1969) demonstrated a further increase in calcium con-

centration in a pre-existing case of hypercalcemia during thiazide administration, and postulated a potentiating effect of the thiazides on the effect of the parathyroid hormone. A similar conclusion was reached by KOPPEL *et al.* (1970) who found that the thiazides induced a rise in the serum calcium concentration in patients on maintenance dialysis.

Although investigations in human subjects have shown that the maximal effect on urinary calcium excretion is not seen until 3–4 days after long-term thiazide administration has started (NASSIM & HIGGINS 1965 YENDT *et al.* 1966 HARRISON & ROSE 1968 PARFITT 1969 BRICKMAN *et al.* 1971), in the present work a significant reduction in urinary calcium is seen in rats 4 hours after a single large intramuscular dose of bendroflumethiazide. The present experiments were undertaken in an attempt to investigate whether such a dose has any effect on the serum calcium concentration which might suggest an extrarenal effect of thiazides in addition to its renal effect on calcium metabolism.

Material and methods

White, male, Wistar rats (Møllegaard Hansens breeding centre), were used in all experiments. Parathyroidectomy (PX) was done by cauterisation in the thiopentemal (thiomebumalum NFN) anaesthesia. Thyroidectomy (TX) with transplantation of the parathyroid glands to the sternocleidal muscle and thyro-parathyroidectomy (TPX) were done surgically under ether anaesthesia. PX, TX and TPX were done at least a week before thiazide administration. Nephrectomy was done by ventral incision under ether anaesthesia. All the blood samples were taken by heart puncture under light ether anaesthesia after 12 hours of fasting. Bendroflumethiazide (centyl®) was used in all the experiments, 1 mg/kg (suspension in sesame oil 1 ‰) given intramuscularly The control rats received sesame oil The dose used was supramaximal for the natriuretic effect (KOBINGER & KATIC 1960). Previous experiments have demonstrated that this dose is also supramaximal with regard to the effect on renal calcium excretion (JØRGENSEN 1971b).

In order to investigate the effect of bendroflumethiazide administered intramuscularly on sodium and calcium excretion in the urine 12 groups of rats, 3 in each group, were loaded orally with 50 ml of water/kg and placed groupwise in metabolic cages. Six of the groups were given thiazide, the other 6 groups served as controls. The urine was collected every hour and analysed for sodium and calcium.

Bovine parathyroid hormone (Para-Thor-Mone, Lilly) 1000 USP units/kg was administered subcutaneously

Analyses.

Calcium in the urine and serum was determined by atomic absorption (Perkin Elmer Atomic Absorption Spectrophotometer 290, acetylene air flame, three slot Boling burner) One per cent lanthanum oxide was used for preparation of standards and dilutions in order to obviat any phosphate interference.

Sodium was determined by flame photometry (Eppendorf propane air flame).

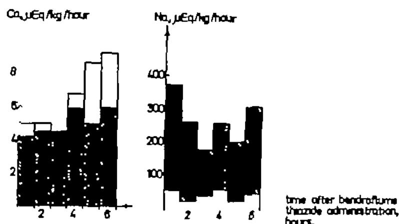


Fig. 1 Renal excretion of sodium and calcium in rats (weight 180 g 205 g) after bendroflumethiazide (centyl®), 1 mg/kg intramuscularly The hatched columns indicate the treated group.

Results

A single intramuscularly given dose of bendroflumethiazide results in a reduced calcium excretion in the urine which is marked after the fourth hour ($P < 0.01$) and still seen during the sixth hour ($P < 0.05$) after thiazide administration. An increased sodium excretion is seen during the whole period ($P < 0.01$) (fig. 1). The increase in renal calcium excretion

Table 1

Effect of a single intramuscular injection of bendroflumethiazide (centyl®) on serum calcium concentration in normal rats. Blood samples are taken 5 hours after thiazide administration. Numbers are given as mean, S.D in brackets.

	Weight g	Serum calcium concentration, mg/100 ml
Thiazide administration n = 8	110 (1)	10.9 (0.5)
Control animals n = 8	110 (2)	10.8 (0.4)
P	> 0.1	> 0.1

Table 2

Effect of a single intramuscular bendroflumethiazide (centyl®) injection on serum calcium concentration in thyroidectomized (TX), parathyroidectomized (PX) and thyro-parathyroidectomized (TPX) rats 5 hours after thiazide administration. Numbers are given as mean with S.D. in brackets.

	Weight (g)	Serum, calcium concentration mg/100 ml before thiazide administration	after thiazide administration	P
TX-rats (n = 12)	176 (7)	9.5 (0.7)	9.4 (0.9)	P > 0.10
PX-rats (n = 15)	121 (6)	5.2 (1.5)	5.4 (1.8)	P > 0.10
TPX-rats (n = 9)	143 (10)	4.3 (0.6)	4.5 (0.5)	P > 0.10

seen in the control groups during the experimental period is probably due to the increased urine flow induced by the water load (WALSER 1961).

No change in the serum calcium concentration after thiazide administration can be seen in any of the rats investigated (table 1 and 2), nor can any potentiating effect of the thiazides on the action of the parathyroid hormone be demonstrated (table 3). The hypercalcaemia following nephrectomy in rats is not changed by concomitant thiazide administration (fig. 2)

Table 3

Effect of parathyroid hormone on serum calcium concentration with and without concomitantly given bendroflumethiazide (centyl®) in thyro-parathyroidectomized rats. Blood samples are taken 5 hours after the injections. All numbers are given as mean with S.D. in brackets.

	Weight (g)	Serum calcium concentration mg/100 ml before thiazide administration	after thiazide	∇ Ca
Parathyroid hormone plus thiazide 9 rats	126 (7)	5.5 (0.6)	8.0 (0.7)	2.4 (0.8)
Parathyroid hormone 9 rats	130 (10)	5.6 (0.9)	8.4 (1.5)	2.9 (1.0)
	> 0.10	> 0.10	> 0.10	> 0.10

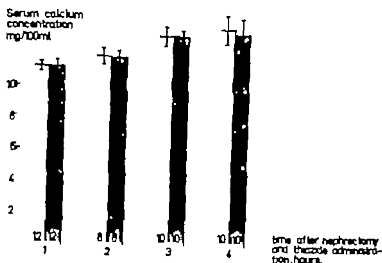


Fig. 2. Effect of bendroflumethiazide (centyl®) 1 mg/kg intramuscularly on serum calcium concentration in rats after bilateral nephrectomy. Bendroflumethiazide is given at the time of nephrectomy. The hatched columns indicate the treated group. The numbers in the columns indicate numbers of rats used. All values are mean ± S.D. Weight of rats 135–165 g.

Discussion

Although several investigations on human subjects (SEITZ & JAWORSKI 1964 YENDT *et al.* 1966 HARRISON & ROSE 1968 PARFITT 1969 KOPPEL *et al.* 1970) and in dogs (PICKLEMAN *et al.* 1969) indicate an extrarenal effect of thiazide diuretics on calcium metabolism, no such effect could be demonstrated in the present experiments either when the thiazide was given alone to normal PX, TX or TPX rats or in combination with parathyroid hormone or in nephrectomized rats. The maximal effect on renal calcium excretion during long term thiazide administration is not seen until the third day at which time the effect on urinary sodium has disappeared. This might be due to the calciuretic effect of the natriuresis (WALSER 1961) antagonizing a possible specific effect of the thiazides on renal calcium excretion. In the present experiments an effect on renal calcium excretion is seen after four hours, but no change in the serum calcium concentration can be detected. This might indicate that a cumulative effect of the thiazides is necessary to reveal an extrarenal effect on bone resorption

The hypercalcaemia seen in rats following nephrectomy is partly due to decreased elimination of the parathyroid hormone, partly to a non-hormonal effect of the nephrectomy on bone resorption both of which are abolished by concomitant acetazolamide administration (WAITE *et al.*

1970 JØRGENSEN 1971a) The fact that no effect of thiazides was found in the present experiments indicates that the carbonic anhydrase inhibiting effect of the thiazide diuretics plays no part in a possible extrarenal effect on calcium metabolism.

The findings of PARFITT (1969) and KOPPEL *et al* (1970) suggesting either a direct stimulating effect of the thiazides on the parathyroid glands or a potentiating effect on the action of the parathyroid hormone is in opposition to the findings of YENDT (1970). YENDT postulated a decreased bone resorption in humans during thiazide administration, as indicated by the urinary hydroxyproline excretion pattern. This observation is in agreement with the results of JØRGENSEN & NIELSEN (1971) who observed decreased calcium mobilisation from the bones of rats during thiazide administration. Futher investigations are needed to clarify the conflicting results of studies on the effect of thiazides on calcium metabolism.

Acknowledgement

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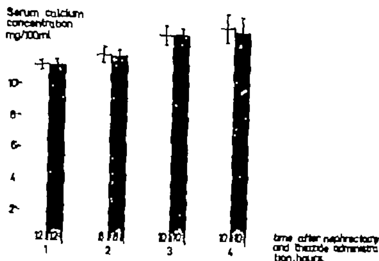


Fig. 2. Effect of bendroflumethiazide (centyl®) 1 mg/kg intramuscularly on serum calcium concentration in rats after bilateral nephrectomy. Bendroflumethiazide is given at the time of nephrectomy. The hatched columns indicate the treated group. The *numbers in the columns indicate numbers of rats used. All values ar* mean ± S.D. Weight of rats 135–165 g.

Discussion

Although several investigations on human subjects (SEITZ & JAWORSKI 1964, YENDT *et al.* 1966, HARRISON & ROSE 1968, PARFITT 1969, KOPPEL *et al.* 1970) and in dogs (PICKLEMAN *et al.* 1969) indicate an extrarenal effect of thiazide diuretics on calcium metabolism, no such effect could be demonstrated in the present experiments either when the thiazide was given alone to normal, PX, TX or TPX rats or in combination with parathyroid hormone or in nephrectomized rats. The maximal effect on renal calcium excretion during long-term thiazide administration is not seen until the third day at which time the effect on urinary sodium has disappeared. This might be due to the calciuretic effect of the natriuresis (WALSER 1961) antagonizing a possible specific effect of the thiazides on renal calcium excretion. In the present experiments an effect on renal calcium excretion is seen after four hours, but no change in the serum calcium concentration can be detected. This might indicate that a cumulative effect of the thiazides is necessary to reveal an extrarenal effect on bone resorption.

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From the Research Laboratories of AB Draco, Lund, Sweden

Adrenoceptors in the Gall bladder

By

Carl G. A. Persson

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Abstract. Effects of sympathomimetics and sympatholytics were studied on cat gall bladder *in vivo* and on cat and human gall bladder strips *in vitro* Isoprenaline 0.1 1 μg/kg and terbutaline 1–10 μg/kg consistently decreased the intraluminal gall bladder pressure *in vivo* Propranolol 1 mg/kg prevented this response. Noradrenaline 1–10 μg/kg also decreased the gall bladder pressure. Isoprenaline (0.08–0.24 μg/ml), terbutaline (0.26–4 4 μg/ml), noradrenaline (0.04–3.2 μg/ml), adrenaline (0.04–1.6 μg/ml) and tyramine (0.08–4 μg/ml) produced the same maximum relaxation in isolated strips from the cat gall bladder. The relaxation was abolished by propranolol 0.1 1 μg/ml but not by practolol 160 μg/ml. Tyramine 2 32 μg/ml was without effect on strips from reserpine-treated cats. After propranolol, noradrenaline and adrenaline consistently produced contraction which was blocked by phenoxybenzamine 0.1 μg/ml. This contraction was much weaker than that produced by acetylcholine 0 08–0.2 μg/ml and cholecystokinin 0.02–0.3 i.u./ml. Different parts of the gall bladder reacted similarly to α- and β-receptor stimulation. About half of the human gall bladders were relaxed by isoprenaline 0 4–1 μg/ml and terbutaline 4–16 μg/ml. Propranolol 5 μg/ml prevented the relaxation. Cat gall bladder contains relaxation mediating β-receptors possibly of the β_2-type and a small population of contraction mediating α-receptors. The adrenergic function may be to counteract cholecystokinin. Human gall bladder preparations can also be relaxed by β-receptor stimulation.

Key-words: Gall bladder – sympathomimetics.

In 1934 IVY reviewed different responses to adrenaline in the gall bladder with the weight of evidence showing that it relaxes the bladder Later studies have confirmed this view and it is now generally accepted that adrenaline as well as stimulation of splanchnic nerves cause gall bladders of animals to relax (reviewed by HALLENBECK 1968) The present paper reports an evaluation of adrenoceptor functions in cat gall bladder *in vivo* and *in vitro* with some experiments on isolated human gall bladder included.

Method

In vivo

The method used is a slight modification of that described by LIEDBERG (1969). Adult cats of both sexes were fasted 24 hours before operation. They were anaesthetized by intraperitoneal injections of 40 mg/kg of pentobarbital sodium (mebumalum NFN) (Abbott) and additional doses of this agent were given intravenously during the experiment. The animal was placed on a thermostat heated table that kept the rectal temperature at 38 . Artificial respiration was given continuously.

Midline laparotomy was performed and the cystic duct was ligated less than 1 cm from the entrance into the common bile duct. Care was taken that the ligature did not disturb blood and nerve supply to the gall bladder.

A saline filled catheter (PE 90–120) with drainage holes was introduced through the fundus of the gall bladder and a purse-string suture held it in a position with as little contact with the bladder as possible. No bile was allowed to drain off the gall bladder during catheterization. The catheter was connected to a pressure transducer (Statham AC 28 P). Gall bladder pressure and in most experiments heart rate obtained from ECG (triggering a tachometer) were recorded on a Grass polygraph model 7 PI. All drugs were given in the inferior v. cava through a catheter introduced through the femoral vein.

In vitro.

Adult cats fasted for 24 hours were anaesthetized (40 mg/kg of pentobarbital, Abbott) and bled. The gall bladder was taken out and gently rinsed free from bile in Krebs solution. Two to four strips 20 × 4 mm were cut circularly above the gall bladder fundus. From two cats strips were also taken from the gall bladder fundus and collum. They were carefully freed from serosal and some mucosal coverings and mounted in a 25 ml organ bath filled with Krebs solution aerated with carbogen (95% O_2, 5% CO_2) and kept at 38 . The initial tension was adjusted to 0.1–0.3 g and isometric tension changes were recorded on a Grass Polygraph (7 PI) via Grass forcedisplacement transducer (Ft 03).

Human gall bladders were obtained from cholecystectomy operations. At operation the gall bladder specimen was transferred to 2 Krebs solution and within an hour thereafter strips were prepared and mounted in organ baths like for the cat preparations. Obviously diseased parts of the gall bladder were not used. The human strips were rather thick and initial tensions of 1–2 g were used. On to three strips were taken from each gall bladder.

Drugs used in the study: (±)-terbutaline sulphate (AB Draco, Lund, Sweden), (±)-isoprenaline chloride (Sigma Chemical Company U.S.A.) (−)-noradrenaline bitartrate (Sigma Chemical Company U.S.A.) (−)-adrenaline bitartrate (Sigma Chemical Company U.S.A.), tyramine chloride (Light and Co. Ltd., England), propranolol chloride (ICI, Ltd., England), practolol chloride (ICI, Ltd., England), phenoxybenzamine chloride (benzylytum NFN) (Smith, Kline and French, England), reserpine (Ciba, Schweiz), karbacholine chloride (Pharmacopoea Nordica), acetylcholine chloride (Calbiochem, U.S.A.) cholecystokinin (Prof. J. E. Jorpes, Karolinska Institutet, Stockholm). The drugs were dissolved in fresh glass-distilled water before each experiment. Isoprenaline, noradrenaline and adrenaline solutions were stabilized with ascorbic acid 0.2 mg/ml. Amounts of the adrenoceptor stimulating agents are expressed as bases or as molar concentrations.

Results

In vivo

Experiments were made in 14 cats. Resting pressure in the gall bladder was 6–14 cm H_2O. The basal pressure usually decreased somewhat during the experiment when relaxing agents were given. No spontaneous activity was recorded but respiratory movements influenced the gall bladder pressure (cf. fig. 1). Twelwe of the cats responded consistently with relaxation to isoprenaline (0.1—1 µg/kg) and to terbutaline (1–10 µg/kg), the effect of terbutaline being of longer duration (fig. 1). The maximum decrease amounted to (1–2 cm) H_2O. In most cats the response to the β-receptor stimulating agents diminished and disappeared completely during the course of the experiment. The contracting response to cholecystokinin (1–2 i.u./kg and acetylcholine (1–3 µg/kg) was however still obtainable. Both terbutalme and isoprenaline induced dose-related responses but no closer evaluation of potency ratio between the compounds was possible due to the decreasing sensitivity. Two cats showing good repeatability of response to the relaxing amines were treated with propranolol 1 mg/kg. 15 min. after this treatment, terbutaline (1–10 µg/kg) and isoprenaline (0.1–1 µg/kg) were without effect. Noradrenaline (1–10 µg/kg) was shown to relax the gall bladder in three cats one of which showed a biphasic response with relaxation followed by small contraction (fig. 1). This cat responded to isoprenaline and terbutaline with relaxation only.

In vitro

Early experiments revealed that the gall bladder strips were more sensitive when freed from serosal coverings and only such preparations are included in the results. Almost all the strips from 17 cats were sensitive to and re-

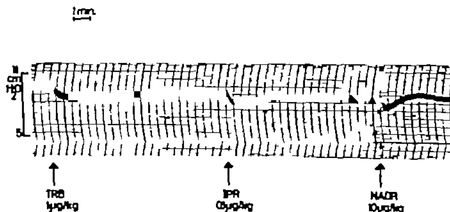


Fig. 1. The effect of terbutaline (TRB), isoprenaline (IPR), and noradrenaline (NADR) on gall bladder pressure in the anaesthetized cat.

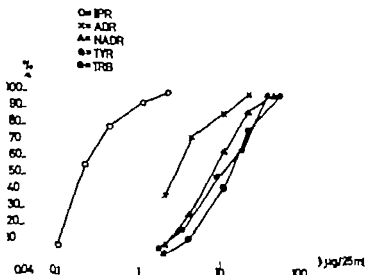


Fig. 2. Cumulative dose-response curves of isoprenaline (IPR) adrenaline (ADR), nor adrenaline (NADR), tyramine (TYR), and terbutaline (TRB) obtained in one isolated gall bladder strip.

laxed by the adrenoceptor stimulating agents. The sensitivity of the strips to these agents usually increased markedly during the first few hours of the ex periment. In about 20% of the strips, a slight spontaneous activity was recorded. This activity was reduced in frequency and amplitude of contraction along with the relaxation produced by the amines. In some of these strips the amplitude increased as the frequency and the basal tension was decreased. A similar finding has been reported for β-receptor mediated responses in the uterus (HAWKINS 1964). Isoprenaline (0.08–0.24 μg/ml), ter butaline (0.26–4 4 μg/ml), noradrenaline (0 1—3.2 μg/ml) adrenaline (0.1–1 6 μg/ml) and tyramine (0.2–4 μg/ml) relaxed the gall bladder and all induced the same degree of maximal relaxation when added cumulatively to the bath. Two strips from one cat acted with contraction to noradrenaline *(1–2 μg/ml) This contraction was potentiated by propranolol.* These strips were only slightly relaxed by larger doses of isoprenaline (1 μg/ml). The slopes of the dose-response curves did not seem to differ between the relaxing compounds (fig. 2)

The cumulative dose response curves of terbutaline and isoprenaline were evaluated on 9 strips from 8 cats. On a molar basis isoprenaline was 36 (range 17–50) times as active as terbutaline based on comparisons made at a level where the dose-response curves showed parallelism (usually ED50 values) The potency ratio seemed to be independent of the sensitivity of the preparation. Propranolol (0 1–1 μg/ml) when added to the bath 15 min. before the sympathomimetic agents, abolished the relaxation Practolol on to

160 μg/ml inhibited the relaxation less than propranolol 0.1 μg/ml (6 strips) The influence of propranolol on noradrenaline and adrenaline was studied in greater detail It was found that these agents consistently produced a contraction of the gall bladder after (0 1–2 μg/ml) of propranolol. The contraction was weak in comparison with carbachol induced contraction and further increase in the dose of noradrenaline and adrenaline changed the contracting response to relaxation again (fig. 3) Phenoxybenzamine (0 1 μg/ml) with 15 min. contact time with the preparation abolished the contracting response to noradrenaline and adrenaline. Noradrenaline after propranolol 0.1 μg/ml contracted the gall bladder muscle more than twice as much as after practolol 160 μg/ml (4 strips).

Acetylcholine (0 08–0.2 μg/ml) and cholecystokinin (0 02–0.3 i.u./ml) consistently contracted the gall bladder strips. The effect of acetylcholine developed rapidly differing from that of cholecystokinin which slowly developed full contraction.

The strips taken from the fundus and collum reacted in the same way as the other strips to sympathomimetic agents. Also from these parts of the gall bladder a weak contracting effect to noradrenaline was obtained after blockade with propranolol.

Four cats were treated with reserpine (3 mg/kg) 16 hours before the gall

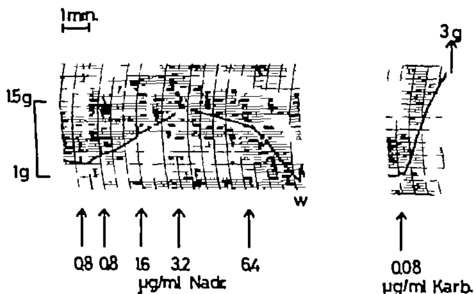


Fig. 3 Recordings from isolated gall bladder strip.

Left The response to increasing doses of noradrenaline (NADR) when propranolol 1 μg/ml had been added to the bath.

Right Carbachol (Karb) contracted the strip much more than did noradrenaline.

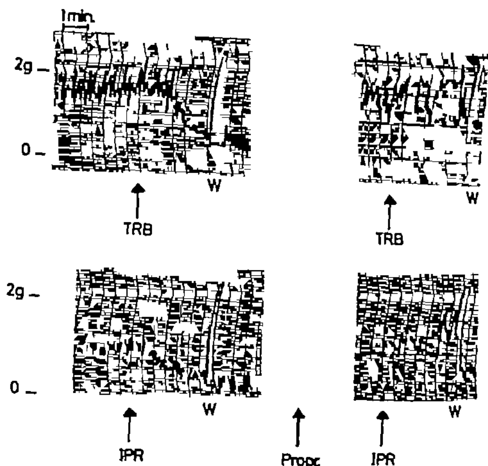


Fig. 4 Recordings from isolated human gall bladder strip The effect of terbutaline (TRB) 8 μg/ml and isoprenaline (IPR) 0.4 μg/ml before and after addition of 4 μg/ml of propranolol (Propr) to the bath.

bladder was taken. Strips from these gall bladders were insensitive to tyramine 2—32 μg/ml while they still were sensitive to noradrenaline. 32 strips from 12 human gall bladders were tested for response to acetylcholine, isoprenaline and terbutaline Strips from 6 respectively 5 gall bladders were relaxed by isoprenaline (0 4–1 μg/ml) and terbutaline (4–16 μg/ml) (fig. 4). The effect began about 1 minute after addition of the drug. The response showed variation in repeatability Two of the strips with consistent responses were treated with propranolol (5 μg/ml) for 15 min. After this treatment, isoprenaline and terbutaline were without effect (fig. 4).

Only two strips failed to contract when acetylcholine (0.2–1 6 μg/ml) was added to the bath. The response to acetylcholine usually occurred after a short delay and consisted of a prompt rise in tension with maximum effect within two minutes.

Discussion

The investigation has shown that the essential effect of sympathomimetic agents on the cat gall bladder is relaxation. This effect is due to β-adrenoceptor activation as evidenced by the ability of propranolol to inhibit the relaxation. The relaxing effect induced by adrenaline and by stimulation of splanchnic nerves as reported by others (reviewed by IVY 1934 and HALLENBECK 1968) can probably also be explained as due to activation of β-receptors in the gall bladder

Isoprenaline was a more potent relaxing agent than both adrenaline and noradrenaline, and phenoxybenzamine did not prevent the relaxation. These findings support the conclusion that sympathomimetic agents relax the gall bladder by activation of β-adrenoceptors. LANDS *et al.* (1966) have classified heart muscle to contain β_1-receptors and bronchi β_2-receptors, and according to this classification terbutaline is selective β_2-receptor stimulating compound (PERSSON & OLSSON 1970) Isoprenaline was 36 times as active on the gall bladder as terbutaline compared on molar basis. The results obtained *in vivo* are in agreement with this potency ratio The ratio differs from that reported for the effect of the two compounds on isolated heart preparations where isoprenaline is about 500 times as active as terbutaline (PERSSON & OLSSON 1970). It is more similar to the ratio reported for bronchi (PERSSON & OLSSON 1970) and sphincter of Oddi (PERSSON 1971) where isoprenaline is about 20 times as active as terbutaline.

The finding that the selective β_1 receptor blocker practolol (LEVY & WILKENFIELD 1969) did not inhibit the β-receptor mediated relaxation of the gall bladder together with the potency ratio found between isoprenaline and terbutaline indicate that the gall bladder contains β-receptors of the β_2-type.

It was clearly shown that noradrenaline and adrenaline were able to contract the gall bladder Since this response was abolished by phenoxybenzamine it is probably an α-receptor mediated effect. It was a very weak effect, however and *in vitro* it could be shown only after β-receptor blockade with propranolol, which makes the functional significance of the α-receptors seem doubtful. However in bronchi where α-receptors mediate weak contractions under similar conditions as in the gall bladder experiments (PERSSON & JOHNSSON 1970), the contracting effect can be markedly potentiated by bacterial endotoxin (SVEDMYR 1971) This finding indicates that the functional importance of the gall bladder α-receptors cannot be ruled out on the basis of the present experiment. Maybe the slight contracting part of the biphasic response to noradrenaline experienced in one *in vivo* experiment can be explained as due to α-receptor stimulation

Experiments on different parts of the gall bladder suggested that the adrenoceptor population did not differ between various parts of the gall

bladder as has been shown to be the case in the urinary bladder (EDVARDSEN & SETEKLEIV 1968).

The gall bladder is innervated with adrenergic fibers (cf. BAUMGARTEN & LANGE 1969). Tyramine relaxed the gall bladder but the relaxing effect was not present in strips from reserpine-treated animals suggesting that the effect of tyramine was mediated through release of noradrenaline from adrenergic nerves. This finding is in agreement with the reports showing that the sympathetic nervous system mediates relaxation of the gall bladder (reviewed by HALLENBECK 1968). Similar experiments with tyramine on the isolated cat sphincter of Oddi (PERSSON 1971) showed that the tyramine mediated effect and the effect of noradrenaline was contraction of the sphincter Cholecystokinin, however relaxed the sphincter and contracted the gall bladder as also shown by many other investigators (see for example MAGEE 1965). The effect of tyramine thus indicates that adrenergic influences may have the function to counteract hormonal (cholecystokinin) smooth muscle actions on the sphincter Oddi and gall bladder This has been suggested for the gall bladder by PALLIN & SKOGLUND (1964) who showed that stimulation of splanchnic nerves diminish the response to cholecystokinin.

In human gall bladder strips it was also found that β-receptor stimulation induced relaxation. This disagrees with the report by MACK & TODD (1968) suggesting that up to 100 μg/ml of isoprenaline was without effect on the isolated human gall bladder If these authors tested isoprenaline only on a few isolated gall bladder strips this might explain the disagreement since some gall bladder preparations were insensitive to isoprenaline and terbutaline. This may in turn be due to the gall bladder not being quite free from disease, or the strips being too thick or perhaps the strips should be in a more contracted state to show relaxation. These factors were not particularly investigated.

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From the Departments of Physiology and Biochemistry
University of Turku, Turku, Finland

Studies on the Control of Glucuronide Synthesis in the Rat Small Intestine

By

E. Hietanen, A. Aitio and O. Hänninen

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Abstract. Gastrointestinal surgery was used in order to determine the role of normal food components, bile and pancreatic secretion in the control of mucosal glucuronide synthesis. Furthermore a few rats were treated either intragastrically or intraperitoneally with phenobarbitone. Both the rate of o-aminophenyl glucuronide synthesis in slices and the activity of UDP glucuronyltransferase in mucosal extracts were studied. In partial jejunectomies no changes were found in glucuronide synthesis when compared with the controls, but in jejunal Thiry Vella loops and in duodenal and jejunal blind sacs the glucuronide synthesis rate was significantly lower In gastro-jejunostomized rats a transient increase in the glucuronide synthesis rate was noted in the gastro-jejunostomy area. Phenobarbitone administration did not increase the small intestinal UDP glucuronyltransferase activity when administered either intraperitoneally or intragastrically The results indicate that the chyme factors are of importance in the control of glucuronide synthesis in the small intestine of the rat, but that phenobarbitone has no effect.

Key-words: Adaptation, physiological – glucuronides – surgery operative.

The activities of UDP glucuronyltransferase (Hänninen *et al.* 1968) and UDP glucose dehydrogenase (Hänninen *et al.* 1966) decrease very rapidly in the mucosa of the small intestine from its oral to its aboral end. This is also reflected in the rate of o-aminophenyl glucuronide synthesis in small intestinal slices (Hänninen *et al.* 1968). This gradient has been explained by *assuming higher levels of enzyme inducers in the chyme of the oral end than* in the aboral end of the small intestine (Hänninen *et al.* 1968).

In order to determine further the role of natural inducers in the control of glucuronide synthesis in the gut, we changed the chyme flow and the flow of bile and pancreatic secretion by gastro-intestinal surgery Since phenobarbitone (phenemalum NFN) has proved to be one of the most *useful* inducers of drug metabolism in the liver (Remmer & Alsleben 1958 Conney

1967 HÄNNINEN & AITIO 1968), it was administered both intraperitoneally or intragastrically in order to see whether it has any inducing action on intestinal *UDP* glucuronyltransferase.

Methods

Adult male Wistar rats (158 in total) were used. The rats were fed *ad libitum*. They were, however fasted one day before and after the operations. The operations were performed under mebumal anaesthesia (50 mg/kg, sombutol® Lääke Oy Turku, Finland). The gastro-jejunostomies with pyloric ligations were made by connecting the jejunum 20 cm from the ligament of Treitz with the antral stomach and by ligating the pylorus with silk suture. *In partial jejunectomies a 20 cm segment of the upper* jejunum was resected. For jejunal Thiry-Vella loops, two successive 10 cm segments were cut 10 cm aborally from the ligament of Treitz and their ends were sutured to the skin. The self-emptying duodenal blind sacs supplied by bile and pancreatic ducts and 20 cm jejunal blind sacs from the upper jejunum were anastomosed by an end-to-side anastomosis to the lower ileum as described by ALTMANN & LEBLOND (1970). The sham-operations were made by cutting the jejunum 20 cm aboral to the ligament of Treitz and by re-anastomosing the ends.

Phenobarbitone was administered intragastrically to one group of rats and intraperitoneally to another group at a dosage of 80 mg/kg for 5 days.

The rats were killed by a blow on the head and bled by cutting the renal vessels. *The samples were collected and the whole wall tissue slices (HARTIALA & RONTU 1955)* and mucosal homogenates in digitonin solution (HÄNNINEN *et al* 1968) were made as described previously. The rate of o-aminophenyl glucuronide synthesis in whole wall slices was determined as described by HARTIALA & RONTU (1955). *p*-Nitrophenol was used as substrate in the determination of the UDP glucuronyltransferase activity of mucosal homogenates (HÄNNINEN *et al.* 1968).

Results

The rate of *o*-aminophenyl glucuronide synthesis in the small intestinal whole wall slices remained at the same level as that in the control rats for eight weeks after partial jejunectomy (fig. 1 A) In the gastro-jejunostomy area the rate of *o*-aminophenyl glucuronide synthesis was increased significantly four weeks after gastro-jejunostomy with pyloric ligation (fig. 1 B). This change was, however reversible since twelve weeks *after the operation* no significant difference could be found as compared with the control rats (fig. 1 B).

In the jejunal Thiry-Vella loops with no access to chyme, the rate of *o*-aminophenyl glucuronide synthesis decreased drastically in 4 weeks (fig. 1 A). *In the wall of the self-emptying jejunal blind sacs* a decrease in *o*-aminophenyl glucuronide synthesis was found 4 weeks after the operation (fig. 2 A) The mucosal UDP glucuronyltransferase activity was also much

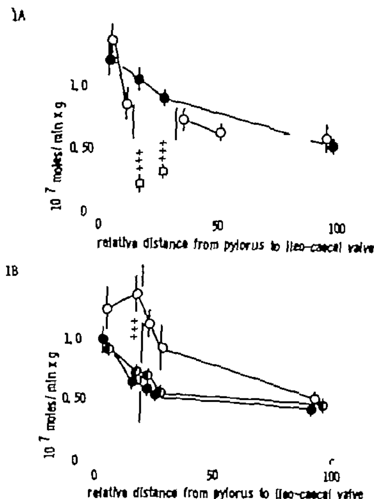
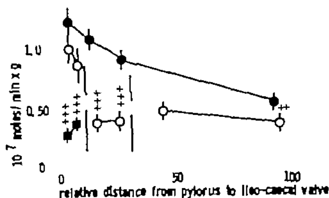


Fig. 1. The rate of o-aminophenyl glucuronide synthesis in whole wall tissue slices calculated on dry weight basis. (A) In the small intestine of the control rats (black dots) 8 weeks after a 20 cm resection of the small intestine (open circles) and in jejunal Thiry-Vella loops (open squares) (29 7 and 6 rats, respectively); (B) In the small intestine of the control rats (black dots), 4 weeks after gastro-jejunostomy with pyloric ligation (open circles) and 12 weeks after gastro-jejunostomy with pyloric ligation (half-open circles) (5 12 and 18 rats, respectively).

Student's t-test has been used to calculate the statistical significance of the differences as compared with the control rats. The following signs are used $-$ ($P > 0.05$), $+$ ($P < 0.05$), $++$ ($P < 0.01$) and $+++$ ($P < 0.001$). The standard errors of the means are given. The distance from the pylorus to the ileo-caecal valve is denoted as 100.

lower in the jejunal blind sacs than in the corresponding area of the control rats (fig. 2 B).

2A



2B

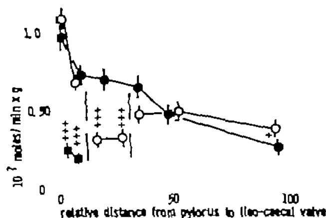


Fig. 2. (A) The rate of *o*-aminophenyl glucuronide synthesis in whole wall tissue slices expressed as the amount of glucuronide synthesized per min. × g of dry tissue in the small intestine of the unoperated control rats (black dots), jejunal blind sacs (open circles) and duodenal blind sacs (black squares). There were 29, 16 and 11 rats, respectively. (B) The UDP glucuronyltransferase activity in the mucosal homogenates of the small intestine expressed as the amount of *p*-nitrophenol bound per min. × g of fresh tissue. Dots, circles and squares as above. There were 10 control rats, 16 jejunal blind sacs and 11 duodenal blind sacs. *For other explanations see fig. 1.*

The duodenal blind sacs with a supply of bile and pancreatic secretion were studied one month after the operations. The rate of *o*-aminophenyl glucuronide synthesis had greatly decreased, especially in the proximal part of the sac (fig. 2 A). Similarly the UDP glucuronyltransferase activity was much lower (fig. 2 B).

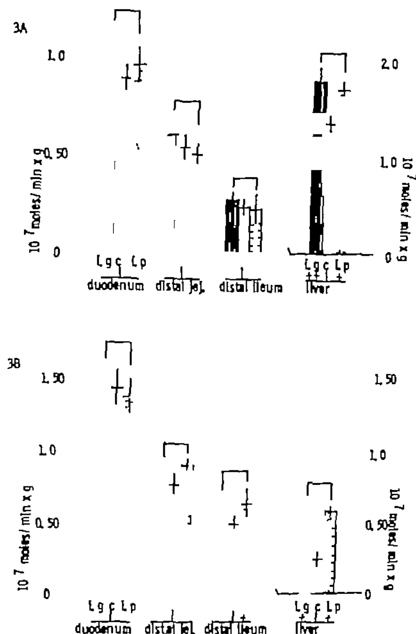


Fig. 3 (A) The UDP glucuronyltransferase activity in the mucosal homogenates of the small intestine and liver expressed as the amount of *p*-nitrophenol bound per min. × g of fresh tissue. Phenobarbitone was administered for 5 days either intragastrically (l. g.) or intraperitoneally (l. p.), (c) represents the control rats. There were 14, 6 and 18 rats, respectively. (B) The *o*-aminophenyl glucuronide synthesis rate in whole wall tissue slices of the small intestine and in the liver slices expressed as the amount of glucuronide synthesized per min. × g of dry tissue. For other explanations see fig. 1

In the sham-operated rats the enzyme pattern of UDP glucuronyltransferase was similar to that in the control rats.

The administration of phenobarbitone did not give rise to any response in the small intestine, as indicated by the unchanged mucosal UDP glucuronyl transferase activity (fig. 3 A) or in the rate of *o*-aminophenyl glucuronide synthesis in the intestinal slices (fig. 3 B) when given either intragastrically or intraperitoneally

In the jejunal Thiry Vella loops and blind sacs, the mucosal fresh weight/cm segment decreased from 32 ± 4 mg to 10–15 ± 3 mg and in the duodenal blind sacs from 54 ± 3 mg to 16 ± 4 mg within one month. In the phenobarbitone treated rats the mucosal fresh weights did not show any significant changes.

In the liver homogenates the UDP glucuronyltransferase activity was increased after intraperitoneal or intragastric administration of phenobarbitone (fig. 3 A) In the liver slices a marked increase in the *o*-aminophenyl glucuronide synthesis rate was found after drug administration (fig. 3 B) The liver fresh weight-body weight ratio increased in six days from 0.040 to 0 056 and 0 057 when the drug was given intraperitoneally and intragastrically respectively

Discussion

The greatly reduced activity in the rate of *o*-aminophenyl glucuronide synthesis in slices from the jejunal Thiry-Vella loops and blind sacs as well as the decreased activity of the UDP glucuronyltransferase in the mucosal homogenates, indicate that the chyme contains factors which affect the rate of glucuronide synthesis. This conclusion is further supported by the increase in glucuronide synthesis in the jejunum after gastro-jejunostomy i. e. when the gastric contents enter the area which normally already deals with partially digested food, components of which have been already absorbed.

The lower glucuronide synthesis rate in duodenal blind sacs shows that bile and pancreatic secretion are of minor importance in the regulation of mucosal glucuronide synthesis. It is obvious that the lack of chyme is the reason for the decreased activity in the mucosal glucuronide synthesis of the duodenal blind sacs.

As shown earlier the length of mucosal villi increased in the gastro-jejunostomy area (REVONTA *et al.* 1970) This is at least partly the reason for the higher enzyme activity due to the higher proportion of active tissue in the slices. The regression of the *o*-aminophenyl glucuronide synthesis rate 12 weeks after gastro-jejunostomy to the control level supports this conclusion, since the hyperplasia also decreases with time (REVONTA *et al* 1970)

In jejunal Thiry Vella loops and blind sacs the mucosal fresh weight/cm segment decreased significantly which indicates marked mucosal atrophy in the absence of chyme. In duodenal blind sacs in spite of bile and pancreatic juice, the fresh weight/cm also fell drastically within one month. This decrease in the mucosal fresh weights is to a great extent caused by atrophy of the mucosal villi. These results indicate the essential role played by chyme in the maintenance of the normal structure and function of the mucosa.

The fact that phenobarbitone did not induce small intestinal *o*-aminophenyl glucuronide synthesis or UDP glucuronyltransferase activity in contrast to the situation found in the liver indicates that the glucuronide synthesis is probably controlled by different mechanisms in these tissues. It is also possible that phenobarbitone itself is not the inducer and that its active metabolite (the inducer?) is not formed in the mucosal cells. Phenobarbitone appears to be a highly liver-specific inducer since the rate of glucuronide synthesis remains unaffected also in the kidney (HÄNNINEN & AITIO 1968). The drug does not induce glucuronide synthesis in the small intestinal mucosa either when entering the epithelial cells from the blood circulation (intraperitoneal administration) or from the luminal side (intragastrical administration).

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From the Department of Pathology Karolinska Institutet, Stockholm, and the Department of Hygiene, Uppsala University Uppsala, Sweden

The Anti-Inflammatory Action of Phenyl-Methyl-Oxadiazole (PMO) An Experimental Study on the Guinea-Pig Trachea

By

Sven E. Dahlgren and Tore Dalhamn

(Received October 21, 1971 Accepted December 14 1971)

Abstract. The anti-inflammatory action of phenyl-methyl-oxadiazole (PMO) was studied in guinea pigs. Inflammation of the respiratory organs was induced chemically by acrolein aerosol. In repeated experimental series, two involving intraperitoneal and one with oral administration of the test solutions, the anti-inflammatory action was found to be statistically significant.

Key-words: Phenyl-methyl-oxadiazole – anti-inflammatory action on guinea pig trachea – *in vivo*

Phenyl-methyl-oxadiazole (PMO) is structurally related to oxolamine (3-phenyl 5-diethyl-aminoethyl-1,2,4 oxadiazole) Oxolamine citrate has an anti-inflammatory and an anti-tussive action as demonstrated clinically by CORNELLA (1960), DEIDDA (1960) and TROPIA (1961) among others, and experimentally by SILVESTRINI & POZZATTI (1960) DAHLGREN & DALHAMN (1966) and DALHAMN & RAUD (1968). In a previous study the authors were able to demonstrate an anti-tussive action of PMO (DAHLGREN & DALHAMN, unpublished results). In the present investigation the anti-inflammatory action of PMO has been tested on experimentally produced inflammation in the respiratory organs of guinea pigs.

Methods

Inflammation was produced in the respiratory organs of guinea pigs. The animals were then exposed to acrolein in a plexiglass cylinder fitted with a Heyer-Piccolo spray (DAHLGREN & DALHAMN 1968). Previous pilot studies had shown that the optimum exposure time and concentration of acrolein were 5 minutes and 5 per cent respectively

The anti-inflammatory properties of PMO and phenylbutazone were studied. Phenylbutazone was used in order to compare the effect of PMO with the known anti-

inflammatory action of this substance (VON RECHENBERG 1961). One group of animals was treated with sodium chloride as control and another group with methocel® the suspending medium for PMO. The studies were repeated on three series of guinea pigs. In the first two series the animals were treated by intraperitoneal injections while in the third group the test solutions were administrated orally.

A group of six guinea pigs served as a control. Three of the animals were killed for examination immediately before the beginning of the exposure of the other groups and three immediately after the exposure periods for the others. None of the control animals showed any significant lesions in their respiratory organs.

In the first two series, the test solutions were injected intraperitoneally immediately after the acrolein exposure. The initial doses for PMO and phenylbutazone were 30 mg per kg body weight (b.w.). Thereafter the injections in all the four groups were repeated eleven times during the following 76 hours (after 3, 6, 9 24, 28, 32, 48, 52, 56, 72, 76 hours). Each repeated dose was 20 mg per kg body weight. The experiments were concluded after 80 hours. In all animals the initially injected volumes of test solutions were 0.15 ml per 100 g b.w. and in the repeated injections 0.10 ml per 100 g b.w. In the third series, after the same type of acrolein exposure, the various test substances were administrated orally. The initial doses were NaCl and methocel 0.15 ml per 100 g b.w. and phenylbutazone and PMO 30 mg per kg b.w. Thereafter the solutions were administrated ten times (after 3, 6, 9 24 28, 32, 48, 52, 56 and 72 hours). The doses being then in the NaCl-group and methocel-group respectively 0.1 ml per 100 g b.w. and 20 mg per kg b.w. in the two other groups. The experiments were stopped after 76 hours.

Animals dying during the course of the experiments were immediately autopsied. The animals which survived the 80-hour or 76-hour limit were then killed and autopsied. The lungs were examined macroscopically weighed and the lungs and trachea fixed in 10 per cent formalin. After fixation, material from the trachea and each lung lobe was taken for histological examination. The tissue was embedded in paraffin, sectioned and stained with van Gieson and with haematoxylin-eosin.

The degree of atelectasis was mainly estimated according to the macroscopic findings while the degree of inflammation was graded exclusively on the histological material.

The degrees of lesions seen at the macroscopic and microscopic examination were graded according to the following scale: 0 = no lesions; 1 = slight lesions; 2 = moderate lesions, and 3 = severe lesions.

The different types of inflammatory lesions in the trachea, bronchi and lung parenchym are shown in figs. 1–5. The examiner had no knowledge of the type of therapy given to the individual animals.

The statistical calculations were performed by analysis of variance. The effects of treatment with PMO phenylbutazone and methocel were compared with those of the sodium chloride treated control animals within each series. The degrees of statistical significance are expressed as P values.

Material

The intraperitoneal studies were repeated in two series of twenty-four guinea pigs each (series 1 and 2). In each series the animals were randomly divided into four groups of six. In the oral (series 3) experiment we used 48 guinea pigs divided into four groups of twelve.

Table 1

Differences in body weight from the beginning of the experiment to the time when the animals died or were killed.

Treatment	Differences in body weight (mean values in gram)		
	Series 1	Series 2	Series 3
PMO	− 6.3	+ 6.7	+ 0.6
Phenylbutazone	−26.7	−21.0	− 5.8
Methocel	− 2.8	−17.5	−10.5
Sodium chloride	−18.5	− 4.0	−22.4

Results

During the experiment most animals lost weight. Only in the PMO treated groups of animals was there a tendency to maintain or even gain body weight (table 1).

When present the inflammatory reaction in the trachea was of the acute desquamating type. With only slight inflammation, an infiltration of granulo-

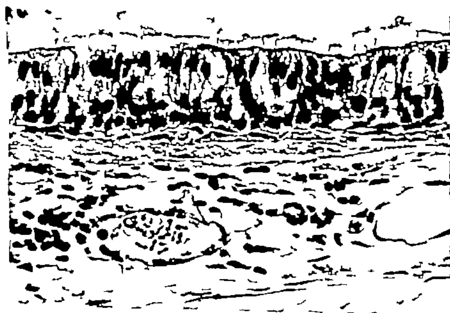


Fig. 1. Normal guinea-pig trachea. The pseudostratified columnar ciliated epithelium is well preserved. Magnification approximately × 500.



Fig. 2. Inflammatory lesions grade 1 in tracheal epithelium. Note loss of cilia, intra-epithelial oedema and loss of goblet cells. Magnification approximately × 500.



Fig. 3. Inflammatory lesions grade 2. No cilia. Moderate inflammatory cell infiltration. Magnification approximately × 500.



Fig. 4 Inflammatory lesion grade 3 Marked inflammatory cell reaction and desquamation of the epithelial cells. Magnification approximately × 500.

Table 2

Inflammatory reaction in the tracheal tissue.

Series no.	Test solution	Number of guinea pigs: Degree of inflammation None (0)	Slight (1)	Moderate (2)	Severe (3)	Total grading of inflammation (mean value)
1	PMO	2	4	0	0	0.7
	Phenylbutazone	1	3	1	1	1.3
	Methocel	1	3	2	*0*	*1.2*
	Sodium chloride	0	2	3	1	1.8
2	PMO	3	3	0	0	0.5
	Phenylbutazone	0	3	3	0	1.5
	Methocel	0	4	2	0	1.3
	Sodium chloride	0	3	3	0	1.5
3	PMO	7	4	0	1	0.6
	Phenylbutazone	4	7	1	0	0.8
	Methocel	1	6	1	4	1.6
	Sodium chloride	0	4	4	4	2.0

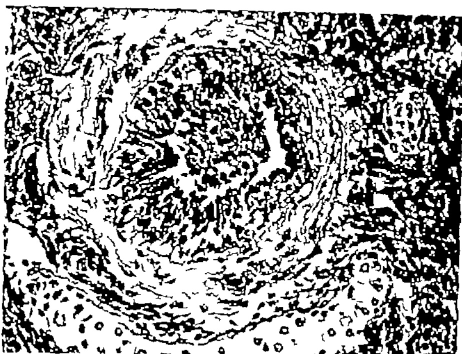


Fig. 5 Moderate inflammatory changes in a medium sized bronchus. The lumen is partly obliterated by desquamated epithelial cells. Magnification approximately × 200.

Table 3

Inflammatory reaction in the bronchi.

Series no.	Test solution	Number of guinea pigs: Degree of inflammation: None (0)	Slight (1)	Moderate (2)	Severe (3)	Total grading of inflammation (mean value)
1	PMO	2	3	1	0	0.8
	Phenylbutazone	1	2	2	1	1.5
	Methocel	1	2	2	1	1.5
	Sodium chloride	0	1	4	1	2.0
2	PMO	2	2	2	0	1.0
	Phenylbutazone	0	5	1	0	1.2
	Methocel	0	4	2	0	1.3
	Sodium chloride	0	2	3	1	1.8
3	PMO	6	4	2	0	0.7
	Phenylbutazone	3	8	1	0	0.8
	Methocel	3	2	4	3	1.6
	Sodium chloride	0	2	5	5	2.3

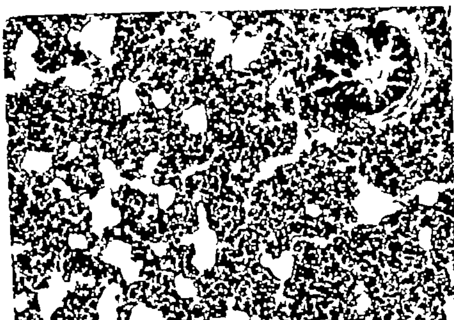


Fig. 6. Moderate inflammatory lesions in lung parenchyma and small bronchi. Slight compensatory emphysema. Magnification approximately × 80.

Table 4

Inflammatory reaction in the lung parenchyma.

Series no.	Test solution	Number of guinea pigs				
		Degree of inflammation				Total grading of inflammation (mean value)
		None (0)	Slight (1)	Moderate (2)	Severe (3)	
1	PMO	2	2	2	0	1.0
	Phenylbutazone	1	2	2	1	1.5
	Methocel	1	2	3	0	1.3
	Sodium chloride	0	4	1	1	1.5
2	PMO	0	6	0	0	1.0
	Phenylbutazone	1	3	2	0	1.2
	Methocel	0	2	3	1	1.8
	Sodium chloride	0	2	4	0	1.7
3	PMO	3	7	2	0	0.9
	Phenylbutazone	3	4	5	0	1.2
	Methocel	1	3	7	1	1.7
	Sodium chloride	0	2	9	1	1.9

Table 5

Degree of atelectasis.

Series no	Test solution	Number of guinea pigs				
		Degree of atelectasis				Total grading of atelectasis (mean value)
		None (0)	Slight (1)	Moderate (2)	Severe (3)	
1	PMO	5	1	0	0	0.2
	Phenylbutazone	3	3	0	0	0.5
	Methocel	3	2	1	0	0.7
	Sodium chloride	1	4	1	0	1.0
2	PMO	5	1	0	0	0.2
	Phenylbutazone	4	2	0	0	0.3
	Methocel	2	4	0	0	0.7
	Sodium chloride	1	5	0	0	0.8
3	PMO	7	5	0	0	0.4
	Phenylbutazone	6	6	0	0	0.5
	Methocel	3	7	2	0	0.9
	Sodium chloride	2	8	2	0	1.0

Table 6

Mean relative lung weight in acrolein exposed animals after treatment with different test-solutions.

Treatment	Mean relative lung weight (g/100 g body weight)		
	Series 1	Series 2	Series 3
PMO	1.23	0.98	0.95
Phenylbutazone	1.30	0.93	0.76
Methocel	0.97	1.12	1.12
Sodium chloride	1.23	0.98	0.95

cytes and interepithelial oedema was found in the tracheal wall. The cilia were destroyed and only a few cells showed remnants of ciliary structure (figs. 1 and 2). In moderate to severe inflammation various degrees of epithelial changes were observed up to the extreme of total desquamation (figs. 3 and 4).

The degree of inflammation of the trachea is listed in table 2. The anti inflammatory action of PMO is statistically significant ($P_1 < 0.02$, $P_2 < 0.02$ and $P_3 < 0.001$) when administered either orally or intraperitoneally

None of the other test solution showed any significant anti-inflammatory

activity when injected intraperitoneally After oral administration phenylbutazone had a statistically significant anti-inflammatory action. The inflammatory changes, in the bronchi (fig. 5), were in principle of the same type as in the trachea. The anti-inflammatory action of the test solutions is listed in table 3 In the first series PMO had a statistically significant anti-inflammatory action as compared with sodium chloride ($P < 0.02$). No significant effect on the degree of inflammation was demonstrated in the second series. In the third series both PMO and phenylbutazone had a marked effect on the inflammation which was statistically significant as compared with sodium chloride ($P < 0.0005$).

The inflammatory lesions in the lung parenchyma appeared as an acute bronchopneumonia, sometimes combined with compensatory emphysema (fig. 6). The results of the anti-inflammatory test are shown in table 4 In all the three series PMO appeared to possess an anti-inflammatory activity This was, however statistically significant only in the second and third experimental series ($P_2 < 0.03$ and $P_3 < 0.0002$)

As judged from the total degree of inflammation in the trachea, bronchi and lung parenchyma, the anti-inflammatory action of PMO is significant in all the series.

In animals with trachea-bronchitis the bronchi were sometimes occluded by inflammatory exudate, causing peripheral atelectasis. The degree of atelectasis in the different groups is seen in table 5 In the first two series the extent of atelectasis was significantly less in the PMO-treated animals than in the sodium chloride controls.

The mean relative lung weights (g/100 g body weight) are listed in table 6 In the first two series the PMO-treated groups had the lowest relative lung weights. In the third series the phenylbutazone animals had a somewhat smaller lung weight than the PMO-treated animals. No statistically significant differences, however could be demonstrated in these experiments.

During the experiments seventeen animals died six in the NaCl-treated animals, seven in the methocel-treated animals and two animals *each* in the PMO and phenylbutazone treated groups.

Discussion

The increasing problem of chronic bronchitis mostly initiated by air pollution and smoking have stimulated a search for drugs which might cure or protect against the disease. We have built up a model system for testing the effects of selected drugs on experimentally produced non-bacterial inflammation in the respiratory tract. The method used produces

Table 5

Degree of atelectasis.

Series no.	Test solution	Number of guinea pigs: Degree of atelectasis: None (0)	Slight (1)	Moderate (2)	Severe (3)	Total grading of atelectasis (mean value)
1	PMO	5	1	0	0	0.2
	Phenylbutazone	3	3	0	0	0.5
	Methocel	3	2	1	0	0.7
	Sodium chloride	1	4	1	0	1.0
2	PMO	5	1	0	0	0.2
	Phenylbutazone	4	2	0	0	0.3
	Methocel	2	4	0	0	0.7
	Sodium chloride	1	5	0	0	0.8
3	PMO	7	5	0	0	0.4
	Phenylbutazone	6	6	0	0	0.5
	Methocel	3	7	2	0	0.9
	Sodium chloride	2	8	2	0	1.0

Table 6

Mean relative lung weight in acrolein exposed animals after treatment with different test-solutions.

Treatment	Mean relative lung weight (g/100 g body weight) Series 1	Series 2	Series 3
PMO	1.23	0.98	0.95
Phenylbutazone	1.30	0.93	0.76
Methacel	0.97	1.12	1.12
Sodium chloride	1.23	0.98	0.95

cytes and interepithelial oedema was found in the tracheal wall. The cilia were destroyed and only a few cells showed remnants of ciliary structure (figs. 1 and 2). In moderate to severe inflammation various degrees of epithelial changes were observed up to the extreme of total desquamation (figs. 3 and 4).

The degree of inflammation of the trachea is listed in table 2. The anti-inflammatory action of PMO is statistically significant ($P_1 < 0.02$, $P_2 < 0.02$ and $P_3 < 0.001$) when administered either orally or intraperitoneally.

None of the other test solution showed any significant anti-inflammatory

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From the Psychopharmacology Research Unit, Aarhus University Psychiatric Institute, State Mental Hospital, 8240 Risskov Denmark
(Chiefs. M. Schou and A. Amdisen)

Adenyl Cyclase Activity in Kidneys of Rats with Lithium-Induced Polyuria

By

Arne Geisler Olaf Wraae and Ole Vendelin Olesen

(Received October 25 1971 Accepted November 18 1971)

Abstract. Rats were made polyuric by the addition of lithium chloride to the food. After three weeks, when the polyuria was constant, the renal adenyl cyclase activity was determined and compared with the activity in kidneys of control rats not given lithium. Adenyl cyclase activities were determined as the amount of cyclic AMP-^{14}C formed from ATP-^{14}C; cyclic AMP was isolated by the method of KRISHNA & BIRNBAUMER (1970). Polyuric rats and control rats did not differ as regards the unstimulated and the fluoride-stimulated adenyl cyclase activities, but the polyuric rats showed a lower vasopressin-induced increase (39 per cent) than did th control rats (69 per cent). The lithium concentration in the incubate was below the limit of detection. Our results indicate that inhibition of the vasopressin-stimulated adenyl cyclase in the kidney is the cause of the vasopressin-resistant polyuria produced by lithium.

Key-words. Adenyl cyclase lithium – vasopressin – polyuria.

During lithium treatment some patients develop a reversible polyuria. The condition resembles diabetes insipidus, but the polyuria is resistant to vasopressin and must therefore be of renal origin (SCHOU 1957 ANGRIST *et al.* 1970).

It is by now well established that vasopressin exerts its water-conserving action on the kidney through the adenyl cyclase catalyzed conversion of adenosine triphosphate (ATP) to adenosine 3 5-monophosphate (cyclic AMP) (JOST & RICKENBERG 1971) In water-loaded rats, where the endogenous production of vasopressin is suppressed, the concentration of cyclic AMP in the kidney tissue is low (SENFT *et al.* 1968) the addition of vasopressin to a kidney homogenate leads to a rise in the concentration of cyclic AMP (CHASE & AURBACH 1968). It is therefore possible that the lack of

response to vasopressin during lithium-induced polyuria is due to an interference with this system.

In vitro addition of lithium to the incubate has been shown to inhibit the vasopressin-stimulated activity of kidney adenyl cyclase (DOUSA & HECHTER 1970a). Without addition of sodium to the incubate, high lithium concentrations, 100–300 mM, are required to produce inhibition, after the addition of sodium inhibition is observed with lithium concentrations of 25 mM. We have approached the problem in a different manner by studying rats made polyuric by prolonged administration of lithium and comparing their kidney adenyl cyclase activity with that of rats not given lithium. Rats are suitable experimental animals for this purpose, because lithium administration with the food in appropriate doses regularly leads to a vasopressin-resistant polyuria (THOMSEN 1970)

Material and Methods

Two groups each of 10 male Wistar rats initially weighing about 200 g were given free access to tap water and a food mixture as described by THOMSEN (1970). In one group the rats were made polyuric by mixing lithium chloride with the food in a concentration of 40 mM per kg dry weight. The other group served as controls. The rats' water intake was followed and used as an indicator of the urine volume. As shown in fig. 1, the rats developed pronounced and relatively constant polyuria three weeks after the beginning of the treatment. At this time the analyses were started so that measurements of kidney denyl cyclase activity could be performed during a steady state of polyuria.

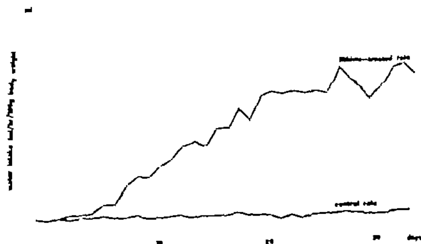


Fig. 1 Water intake of control rats and rats given lithium with the food.

On the same day one kidney from a polyuric rat and one from a control rat were rapidly removed under ether anaesthesia. All subsequent operations were carried out at 0 . One half of each kidney was homogenized in a glass homogenizer for one minute in 4 ml of 50 mM Tris-HCl buffer pH 7.4. The homogenate was centrifuged at 2,200 × g for 10 minutes. The supernatant was decanted, the sediment resuspended in 4 ml of Tris buffer and then recentrifuged. The sediment was resuspended in 4 ml of Tris buffer with 20 mM theophylline.

Three assays were carried out in duplicate for each kidney: i.e. the unstimulated adenyl cyclase activity, the vasopressin-stimulated activity, and the fluoride-stimulated activity.

200µl of the enzyme suspension was used for each assay. Synthetic L-arginine-vasopressin and sodium fluoride dissolved in Tris buffer were added to the appropriate tubes. Thereafter a solution of ATP-8-[14]C, non-labelled ATP and cyclic AMP was mixed with a solution of $MgCl_2$ and added to all the tubes; the substances were dissolved in Tris buffer. The final incubation mixture had a volume of 400 µl and the following composition. ATP-8-[14]C 4 mM (specific activity 0.625 mci/mmol), $MgCl_2$ 8 mM, cyclic AMP 0.57 mM, theophylline 10 mM, and 50 mM Tris buffer pH 7.4 at 30 . The final concentration of L-arginine-vasopressin was 10^{-7} M and that of NaF 10 mM. In agreement with Dousa *et al.* (1970) maximum stimulation with vasopressin was obtained over a broad concentration range. The protein concentration varied between 2 and 3 mg/ml as estimated by the method of Lowry *et al.* (1951).

The mixture was incubated under gentle shaking in a water bath at 30 for 15 minutes. The reaction was stopped by placing the tubes in hot water at 80 for 3 minutes. In the blanks, the enzyme was destroyed by placing the tubes in hot water at 80 for 3 minutes before the addition of the substrate.

The isolation of cyclic AMP from other radioactive products in the reaction mixture was carried out essentially as described by Krishna & Birnbaumer 1970. The recovery of cyclic AMP, determined spectrophotometrically at 259 nm, was about 85 per cent.

Cyclic AMP-[14]C was measured by liquid scintillation.

Lithium concentrations were determined by flame emission spectrophotometry (Amdisen 1967).

Results

The results are shown in table 1. All values are means of duplicate determinations; the relative standard deviation was 5.1 %. Adenyl cyclase activities are expressed as pmol of cyclic AMP formed per mg protein and 15 minutes. No difference was found between the unstimulated adenyl cyclase activities of the control rats and polyuric rats. In contrast, the average vasopressin-stimulated activity was lower in the polyuric than in the control rats, and the difference was statistically significant ($P < 0.01$). The polyuric rats had a higher fluoride-stimulated adenyl cyclase activity than the controls, but this difference was not statistically significant.

Fig. 2 shows for each experiment the vasopressin-induced increase in adenyl cyclase activity expressed as per cent of the simultaneously determined unstimulated activity. In nine experiments the increase was lower

Table 1

Unstimulated, vasopressin-stimulated, and fluoride-stimulated adenyl cyclase activities in polyuric and control rats. Adenyl cyclase activities are expressed as pmol of cyclic AMP formed per mg protein and 15 minutes.

Exp. No.	Unstimulated activity		Vasopressin-stimulated activity		Fluoride-stimulated activity	
	Polyuric rats	Control rats	Polyuric rats	Control rats	Polyuric rats	Control rats
	pmol cyclic AMP/mg protein/15 min					
1	121	95	159	170		
2	88	96	118	196		
3	82	89	119	146		
4	90	96	128	165		
5	96	112	157	183		
6	*102*	93	*142*	*142*	*1080*	*860*
7	81	77	107	122	1140	980
8	110	126	121	198	1570	1040
9	113	126	166	224	1280	1220
10	116	128	172	214	1600	1350
Mean	100	104	139	176	1330	1090

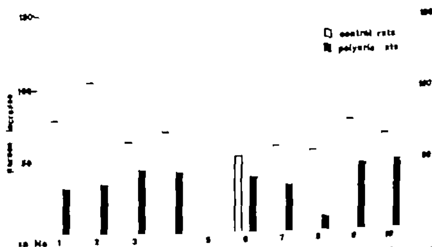


Fig. 2. Vasopressin-induced increases in the adenyl cyclase activity expressed as per cent of the simultaneously determined unstimulated activity. White columns indicate control rats and black columns polyuric rats.

in the polyuric than in the control rats while in one there was no difference. The mean increase over the unstimulated activity was 69 / in the control rats and 39 / in the polyuric rats ($P < 0.001$).

A few experiments were carried out in which lithium chloride was added to incubates with enzyme preparation from control rats. An inhibition of the vasopressin-stimulated activity was found only when the lithium concentration in the incubation mixture exceeded 100 mM.

At the time when the experiments were carried out, the rats with lithium-induced polyuria had serum-lithium concentrations of 0 6–0 8 mM. The lithium concentration in the kidneys was in the order of 1 mM per kg wet weight, but the preparation of the particulate fraction involved washing procedures which removed practically all the lithium. In the final incubation mixture the lithium concentration was below that which could be detected with the method used, i. e. less than 0 01 mM.

Discussion

A decreased formation of cyclic AMP as found in our polyuric rats, might result from (a) an increased ATPase activity (b) an increased phosphodiesterase activity (c) a decreased synthesis of adenyl cyclase, or (d) an inhibition of the vasopressin-stimulated adenyl cyclase activity None of these possibilities can be definitively excluded, but all in all we consider the first three possibilities unlikely for the following reasons (a) ATP was present in excess in the incubate (b) the system contained theophylline and cyclic AMP in concentrations which exert strong inhibitory activity on phosphodiesterase (WEISS & COSTA 1968), and (c) the unstimulated and fluoride-stimulated adenyl cyclase activities were not lowered in the polyuric rats. An inhibition of the vasopressin-stimulated adenyl cyclase activity is accordingly the most likely explanation for our findings.

We have confirmed DOUSA & HECHTER S (1970a) observation that *in vitro* addition of lithium in high concentration leads to inhibition of the vasopressin-stimulated adenyl cyclase activity in the kidney Similar inhibitions of hormone-stimulated adenyl cyclase activities after *in vitro* additions of lithium have been observed in adipose tissue (BIRNBAUMER *et al.* 1969), thyroid (BURKE 1970) and brain (DOUSA & HECHTER 1970b). Under these circumstances the fluoride-stimulated adenyl cyclase activity is also inhibited.

The inhibition which we have found in polyuric rats seems, however to be an entirely different phenomenon. The inhibition does not affect the fluoride-stimulated activity and it could be demonstrated in a system where the lithium concentration is too low for quantitative determination. The inhibition therefore can hardly have been due to the presence of the lithium

ion itself, but must result from metabolic alterations in the tissue induced by the pre treatment with lithium.

The exact site and mechanism of the inhibition are not known. Nor can we as yet definitely conclude that the polyuria is caused by this inhibition although this seems likely Studies are under way to examine these questions in more detail.

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Dr Ivar Øye and co-workers, Institute of Pharmacology University of Oslo, Blindern, Oslo, Norway kindly provided helpful information concerning the assay of adenyl cyclase.

The authors wish to acknowledge the skilful technical assistance of Mrs. Nana Balslev in carrying out this work.

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From the Department of Pharmacology University of Copenhagen,
20 Juliane Maries Vej, DK 2100 Copenhagen Ø Denmark

Cortisol Distribution and Metabolism in Guinea Pigs after High Doses or Treatment for Three Weeks with Indomethacin, Phenylbutazone or Chloroquine

By

Jens Aas Jansen and Jens Schou

(Received October 29 1971 Accepted November 18, 1971)

Abstract. Guinea pigs were pretreated for three days with two daily doses intraperitoneally of phenylbutazone 100 mg/kg (P) or indomethacin 100 mg/kg (I – 100 × 6), or for three weeks with daily single intraperitoneal injections of indomethacin 20 mg/kg (I – 20 × 21) or chloroquine 35 mg/kg (C). P increased the total plasma concentration of cortisol from 441 ± 39 to 658 ± 85 ng/ml ($P < 0.05$) while there were no significant changes in skin cortisol (101 ± 18 to 163 ± 24 ng/g) or plasma ultrafiltrate cortisol (119 ± 12 to 179 ± 45). I – 100 × 6 did not significantly change the cortisol concentrations in the plasma 630 ± 105, skin 155 ± 60 or ultrafiltrat 184 ± 62. I – 20 × 21 and C caused no change in the cortisol values. The concentration of cortisol in the skin, however is well correlated to the concentration of freely diffusible cortisol in the plasma, and for I–100 × 6 there was good correlation between the concentration of the drug in the plasma and the percentage of ultrafiltrable cortisol in the plasma. None of the drug treatments changed the plasma *half-life* ($t_{1/2}$) of exogenous 4-[14]C-cortisol significantly I and C did not change the apparent volume of cortisol distribution, whil P tended to increase the volume. The results do not support the theory of a displacement of cortisol from the plasma protein as basis for the antirheumatic effect of the drugs.

Key-words: Non-steroidal antirheumatics – cortisol distribution – cortisol metabolism – guinea pigs – chloroquine.

In a previous publication the distribution and metabolism of cortisol was examined in guinea pigs after pretreatment for 3 days with non-steroidal antirheumatic drugs (JANSEN & SCHOU 1971). Phenylbutazone (24 mg/kg twice daily for 3 days) tended to decrease the concentrations of cortisol in the plasma, plasma ultrafiltrate and skin and this was in agreement with a tendency to increase the apparent distribution volume (V_d) for intravenously administered (4-[14]C)-cortisol. Indomethacin (8 mg/kg twice daily for 3 days) did not significantly affect the cortisol concentrations, half-life or distribution volume. Recently however very low concentrations of cortisol in the

skin were demonstrated in rheumatic patients after more than 3 weeks of indomethacin therapy (HVIDBERG *et al.* 1971) while there was no correlation between the free fraction of plasma cortisol and the concentration of cortisol in the tissue.

The above mentioned observations led to the present study in which distribution volume and half-life of cortisol, and the concentrations of the hormone in the plasma, plasma ultrafiltrate and skin were measured in guinea pigs after high doses of phenylbutazone and indomethacin, and after prolonged treatment with the latter drug. In the study a group pretreated with chloroquine was also included as this drug has antirheumatic effects and is highly protein bound in the plasma.

Materials and Methods

Young mature white male guinea pigs weighing 350–450 g were used.
Drug treatment was given until 8 a.m. on the day of the experiments. The following treatment schedules were used.

Three days treatment was given by intraperitoneal injections in doses of 12 ml/kg twice daily for three days, containing the following doses per injection in saline (0.9% w/v): Saline 0.9% w/v (controls); phenylbutazone 100 mg/kg, and indomethacin 100 mg/kg.

Three weeks treatment was given by single daily intraperitoneal injections of 10 ml/kg saline (0.9% w/v) for three weeks, containing the following single doses: Saline 0.9% w/v (controls) indomethacin 20 mg/kg, and chloroquine 35 mg/kg.

Endogenous cortisol in plasma and skin.
The animals were stunned by a blow on the neck approximately 2 hours after the last drug injection. Immediately after this 10 ml blood was sampled by heart puncture into heparinized centrifuge tubes after which the guinea pigs were completely bled. The pelt on the lower part of the back was cut with an electric clipper and a skin sample (corium and subcutaneous tissue) was excised. The blood was spun and the plasma and tissue were immediately used for analytical procedure.

The *concentration of cortisol* was determined in 50 µl plasma, 200 µl ultrafiltrate of plasma and 50–100 mg tissue by the method of JANSEN *et al.* (1967). By means of thin-layer chromatography it has been shown that only cortisol is measured by this method, when applied to the skin and plasma from control guinea pigs. The plasma ultrafiltrate was obtained by means of a Visking® cellophane membrane (average pore diameter 24 Å) at 37°, pressure 1 kg/cm², in an atmosphere of 96% O_2 and 4% CO_2 according to the method of AMES & SAKANOUE (1964). Approximately 500 µl ultrafiltrate was collected from 2.5 ml plasma. If the Heller test was positive the ultrafiltrate was discarded.

Half-life and distribution volume for ¹⁴C-cortisol.
The technique used has been described previously (JANSEN *et al.* 1970). Briefly 500 nci 4-¹⁴C-cortisol (specific activity 156 µci/mg) in 100 µl 10% v/v ethanol in water

was injected intravenously into unanaesthetized animals. The blood was sampled by heart puncture from groups of guinea pigs stunned 40, 80 and 160 min. after the labelled injections. For the determination of 4-^{14}C-cortisol, 1 ml plasma was washed with 5 ml hexane and 6 ml dichloromethane.

Five ml of the dichloromethane containing cortisol and other steroids was evaporated to dryness and re-dissolved in 200 µl dichloromethane for paper chromatography using DECKX *et al.* (1964) solvent system "H$_1$" to separate cortisol from labelled metabolites. The amount of labelled cortisol was determined by paper strip radiochromatogram scanning (Packard Model 7201) in relation to standard amounts of 1 nci 4-^{14}C-cortisol carried through the complete procedure.

Using the logarithms of the plasma concentrations of 4-^{14}C-cortisol after 40, 80, and 160 min., the regression line for the mono-exponential decrease was calculated for the control group and the five experimental groups. The linear functions allowed the determination of the half-lives ($t_{1/2}$) and the apparent volumes of distribution (extrapolation of the line to t_0)

Phenylbutazone and indomethacin in plasma.

The concentration of phenylbutazone and indometracin in the plasma were determined according to the methods of FUCHS (1965) and HVIDBERG *et al.* (1972) respectively

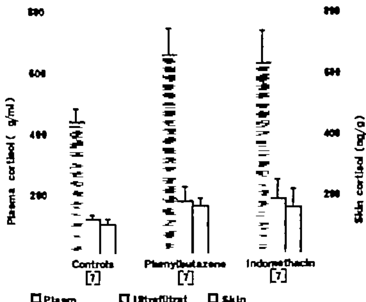


Fig. 1 The concentrations of cortisol in the plasma, plasma ultrafiltrate and skin of guinea pigs after treatment for three days with two daily doses of saline (controls), phenylbutazone or indomethacin, 100 mg/kg/dose for both drugs. Vertical bars indicate S. E. M. Figures in brackets number of experiments. Only the concentration of cortisol in the plasma of the phenylbutazone group was statistically different from the control value ($P < 0.05$).

Results

The concentration of cortisol in the plasma, plasma ultrafiltrate and in the skin of the three days/high dose treatment groups are shown in fig. 1. Only in the phenylbutazone group was the total plasma concentration significantly increased in comparison to the control value ($P < 0.05$) while none of the other experimental values differed significantly from the control levels.

The concentrations of cortisol in the plasma, plasma ultrafiltrate and skin of the three weeks/treatment experiments are shown in fig. 2. None of the concentrations observed after chloroquine or indomethacin treatment showed significant deviations from the comparable control group values.

The concentrations of cortisol in the skin is well correlated to the concentration of freely diffusible cortisol in the plasma. This is demonstrated in fig. 3 in which every single pair of values for cortisol in plasma ultrafiltrate and skin are used. Six pairs of values are lacking, because the ultrafiltrates had to be discarded.

The concentrations of phenylbutazone and indomethacin were measured in the plasma of the ultimate blood sample from each experimental animal to find out whether there was a correlation between the drug concentration and the ratio bound/unbound cortisol in the plasma. After phenylbutazone 100 mg/kg twice daily for 3 days the plasma concentration was 188 ± 14

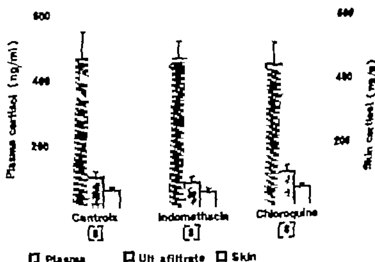


Fig. 2. The concentration of cortisol in the plasma, plasma ultrafiltrate and skin of guinea pigs after treatment for three weeks with daily injections of saline (controls), indomethacin (20 mg/kg/day) or chloroquine (35 mg/kg/day). Vertical bars indicate S. E. M. Figures in brackets indicate number of experiments.

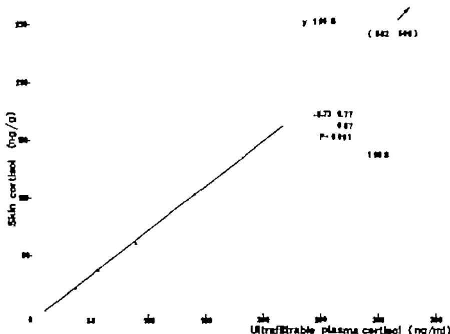


Fig. 3. The concentration of cortisol in the skin (ordinate, ng/g) plotted against the concentration of ultrafiltrable cortisol in the plasma (beciss, ng/ml) The regression line calculated by the method of least squares and the lines for the regression line ± 1.96 $S_{y.x}$ (including 95% of a normal distribution) are indicated in the figure.

Signs

Short term/high dose	*Long term/low dose*
○ controls	● controls
□ indomethacin	■ indomethacin
△ phenylbutazone	▲ chloroquine

µg/ml (n = 8). No correlation was found between the phenylbutazone concentration and the ratio bound/unbound cortisol in the plasma. For the indomethacin group, however the percentage of ultrafiltrable cortisol in the plasma increased with increasing concentrations of indomethacin (table 1) and an analysis of regression also revealed a fairly good correlation ($y = 13.8 + 0.46\ x$, $r = 0.99$ $P < 0.001$).

The plasma half-life ($t_{1/2}$) and the apparent volume of distribution (V_d) of exogenous 4-^{14}C-cortisol are shown in table 2.

The significance of the differences between the half-lives was tested by comparing the slopes of the regression lines, and the differences between the distribution volumes by comparing the segments on the y-axis to time t_o. None of the differences were significant.

Results

The concentration of cortisol in the plasma, plasma ultrafiltrate and in the skin of the three days/high dose treatment groups are shown in fig. 1. Only in the phenylbutazone group was the total plasma concentration significantly increased in comparison to the control value ($P < 0.05$) while none of the other experimental values differed significantly from the control levels.

The concentrations of cortisol in the plasma, plasma ultrafiltrate and skin of the three weeks/treatment experiments are shown in fig. 2. None of the concentrations observed after chloroquine or indomethacin treatment showed significant deviations from the comparable control group values.

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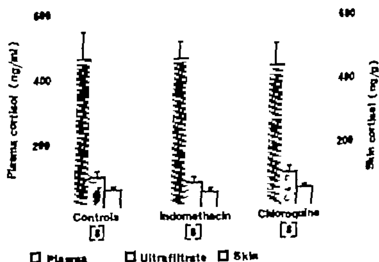


Fig. 2. The concentration of cortisol in the plasma, plasma ultrafiltrate and skin of guinea pigs after treatment for three weeks with daily injections of saline (controls), indomethacin (20 mg/kg/day) or chloroquine (35 mg/kg/day). Vertical bars indicate S.E.M. Figures in brackets indicate number of experiments.

with high doses of phenylbutazone (100 mg/kg twice daily for three days) the cortisol value in the plasma is significantly higher than in the control group. It should be noted that the apparent volume of cortisol distribution tended to increase at both dose levels.

Indomethacin did not affect the half-life or volume of distribution for cortisol either in the present or in the previous investigation (JANSEN & SCHOU 1971). The concentrations of cortisol in plasma and tissue after three days/high dose indomethacin treatment, are at the same levels as for the phenylbutazone treated animals, pointing to a stress induced increase in cortisol secretion. In this connection it should be noted that very high concentrations of indomethacin in the plasma seem to increase the ultrafiltrable fraction of cortisol in the plasma. It should also be noted that the indomethacin concentrations are higher than could be expected with the therapeutic use of the drug (HVIDBERG *et al* 1972) and higher than the concentrations used for our *in vitro* studies on the possible interaction of indomethacin with the protein binding of cortisol (HVIDBERG *et al* 1971). The three weeks/low dose treatment with indomethacin left the cortisol values unchanged, as was also found with chloroquine treatment. In summary there are no indications of any displacement of cortisol from protein binding sites by any of the drugs investigated (conf. JANSEN & SCHOU 1971) except for very high (and toxic?) concentrations of indomethacin. High and apparently toxic doses of phenylbutazone increased the concentrations of cortisol in the plasma, while indomethacin and sodium salicylate tended to have this effect. This could be due to stress induced hypersecretion of cortisol or a direct effect of the drugs as postulated for salicylate by some investigators.

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From the Department of Pharmacology, University of Bergen Medical School,
Haukelandsveien 10, 5000 Bergen, Norway

The *in Vivo* Effect of Tropolone on Dopamine Metabolism and the Catechol-O-Methyl Transferase Activity in the Striatum of the Rat

By

Ole Jacob Broch, Jr.

(Received November 2, 1971; Accepted November 26, 1971)

Abstract: The effect of tropolone (100 mg/kg intraperitoneally) on dopamine metabolism in the corpus striatum of rats was studied. The catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO) activities were measured as were the concentrations of dopamine (DA) and its metabolites 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The amine synthesis was estimated by measuring the accumulation of radioactive metabolites after a constant rate infusion of ^{14}C-tyrosine in tropolone-treated and normal rats. In the striatum the tissue concentration of tropolone was reduced by 90% during the first 2.5 hrs period. Tropolone caused a rapid reduction in the methylated metabolites (3-MT and HVA) while the concentrations of DA and its deaminated metabolite DOPAC were increased. The results from the studies with ^{14}C-tyrosine suggested a reduced DA synthesis rate. All metabolite concentrations returned to normal within 24 hours. After a 20 minutes infusion with ^{14}C-tyrosine a higher radioactivity was found in the fractions of the metabolic end products than in the amine fractions. This finding might indicate an initial turnover of DA in order of minutes and may be a reflection of a multicompartment system for amine storage.

Key words: Catechol-O-methyl transferase – tropolone – corpus striatum – dopamine metabolism.

O-Methylation is an important mechanism for the inactivation of catecholamines (Axelrod *et al.* 1958). The reaction is catalyzed by catechol-O-methyl transferase (COMT). A pharmacological effect on adrenergic activity would be expected after inhibition of this enzyme. No ideal inhibitors of the enzyme are available. They are either metabolized by the enzyme like pyrogallol (Archer *et al.* 1960), or they are short-acting owing to rapid removal. Tropolone and its derivatives belong to the latter group, as no evidence has been found for the *in vivo* O-methylation of these compounds.

Many derivatives of tropolone have been tried as COMT inhibitors (Belleau & Burba 1963; Carlsson *et al.* 1963; Ross & Haljasmaa 1964;

MURPHY *et al.* 1969) but the differences in their actions seem to be relatively small. A convenient method for studying O-methylation is to measure the *in vivo* concentrations of O-methylated metabolites (MURPHY *et al.* 1969). The inhibition may also be studied *in vitro* (BELLEAU & BURBA 1963). To establish whether the drugs act only by inhibiting the enzyme it is necessary to use both methods.

The present report deals with the effects of single injections of tropolone on the following parameters of the rat striatum: COMT and monoamine oxidase (MAO) activities, changes in concentrations of dopamine (DA) and its metabolites and some relative estimations on amine synthesis rate.

Materials and methods

Animals.

Albino Wistar rats of either sex weighing 160–250 g were used in all experiments. The animals were kept in groups of 3 and given free supply of food and water. The rats were killed by decapitation at different times after the injection of tropolone. The brains were removed immediately and the striata dissected free and frozen on dry ice for amine and phenolic acid determinations or stored on ice for enzyme assay. The enzyme activity was measured within an hour while the tissues for chemical analyses were stored at −20° for not more than 4 days.

Drugs

Tropolone (Aldrich Chem. Comp.) was dissolved in distilled water 20 mg/ml, and given intraperitoneally in a dose of 100 mg/kg.

^{14}C Tyrosine (The Radiochemical Centre), specific activity 513 mci/mM, uniformly labelled, was given intraperitoneally by infusion in a dose of 50 μci in 0.8 ml saline, using an infusion pump with a constant rate of 2.25 ml/hr. During the infusion the animals were gently held by hand. By this means the animals were kept completely calm. The infusion was started 10 minutes before the stated times for the tropolone injections.

Assays for catecholamines and phenolic acids.

The striata from three animals were pooled. The amines were acetylated and separated by paper chromatography before the spectrophotofluorimetric determinations (CRAWFORD & YATES 1970). Homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in the striatum according to GULDBERG & YATES (1969). All determinations were corrected for their recoveries which were: (Percentage means of 5 experiments ± S.D.) DA: 83 ± 8; 3-methoxytyramine (3-MT): 25 ± 10; DOPAC: 72 ± 16 and HVA: 62 ± 6.

Determination of metabolites from ^{14}C-tyrosine

The animals were killed immediately after the infusion of ^{14}C-tyrosine and the striata were frozen and stored for analysis. The striata were homogenized in 2 ml 0.4 N perchloric acid. One animal was used for each determination. The extracts were treated according to the procedure of CRAWFORD & YATES (1970) for amines and phenolic acids with the exception that the acids were extracted twice with 10 ml ethyl ether in-

stead of ethyl acetate to facilitate evaporation. Th ether extracts were washed with 5 ml 0.1 N-HCl saturated with NaCl to remove any residue of tyrosine and then chromatographed on paper for 5 hours as described by BROCK & GULDBERG (1971). The chromatograms of the amines and the phenols were cut according to the corresponding markers, and the paper put directly into scintillation vials and counted for 20 min. in a Packard Tri Carb spectrometer The total radioactivity was determined in 0.1 ml of the aqueous extract before extraction of the acids.

The radioactivity (c.p.m.) in the different fractions was divided by the total activity and the amount of metabolized tyrosine expressed as per thousand of total activity The values were not corrected for recoveries, as this was fairly constant for the various substances, with the exception of 3-methoxytyramine.

Estimation of tropolone.

It was found that tropolone gave a spontaneous fluorescence at the wavelengths: 310 mμ (activation) and 400 mμ (fluorescence), and since it behaved like phenol in the extraction procedures, it could be estimated quantitatively Brain samples were homogenized in 0.4 N perchloric acid, centrifuged and the perchloric cid precipitated by adjusting the pH to 6 with 1 N followed by 5 N – KOH. Th precipitate was removed by centrifugation and the tropolone extracted from the supernatant in 10 ml chloroform. At this pH neither the carboxylic acids nor the amines will be extracted into chloroform. The tropolone was re-extracted into 1.5 ml of 0.1 N–NaOH. An aliquot, 1 ml, of the aqueous extract was neutralized with 0.5 ml 0.2 N–HCl after which then 1 ml 0.5 N phosphate buffer pH 6.0, was added. The samples were read in an Aminco-Bowman spectrophotofluorimeter at 310/400 mμ. The fluorescence was found to be extremely pH dependent with maximum at 6 0. A brain sample from non treated animal was used as a blank. The recovery of a tropolone standard added to the homogenate was about 50%

Enzyme determinations.

MAO activity was measured by the method of SNYDER & HENDLEY (1968). COMT was measured according to a previously published method (BROCH & GULDBERG 1971). For the enzyme determinations the tissue was homogenized in 1 ml 0.1 N phosphat buffer pH 7.8, to each 100 mg of tissue. The homogenates were used undiluted for COMT and diluted 1:20 for MAO

Statistical calculations

All values in the tables are the means with their S.E.M. and the number of experiments in brackets. The Lord's two sample test was used for paired results (tables 2 and 4) and the Student's t-test for unpaired (tables 1 and 3). P values of less than 0.05 were considered statistically significant.

Results

The in vitro inhibition of COMT by tropolone

The kinetics of COMT inhibition by tropolone was determined *in vitro* in tissues obtained from the thalamo-striatum and the brain stem. The K_m of the enzyme in crude homogenates using DOPAC as a substrate, was deter mined as 3.5×10^{-4}M. The inhibition constant, K_i, for tropolone was 3.0×10^{-6}M and the inhibition was shown to be competitive.

Table 1

Tropolone concentration and COMT activity in the rat striatum. Dose of tropolone: 100 mg/kg intraperitoneally. Mean ± S.E.M. (no. of experiments)

Hours after injection	Tropolone concentr. µg/g	COMT activity nmol/hr/g	COMT % activity	COMT % activity calc. from tropolone conc.
0	0	44 ± 2.1 (10)	100	
0.5	21 ± 3.4 (5)			
1	19 ± 5.1 (5)	32 ± 4.9 (5)*	73	45
1.5	11 ± 2.2 (4)			
2.5	3.5 ± 1.0 (7)	34 ± 4.1 (5)*	77	81
24	0.3 ± 0.15 (5)	40 ± 8.4 (4)	91	98

Significantly different from 0 hr ($P < 0.05$) (Student's t-test).
Calculation based on K_m (substrate: DOPAC, 3.5×10^{-4}M), concentration of tropolone and k_i for tropolone (3×10^{-6}M).

The relation between the COMT activity in the striatum and the concentration of tropolone

After an intraperitoneal injection of tropolone, the substance penetrated rapidly into the brain. Maximal concentrations were reached in 0.5 hr (table 1). In the following 2–3 hrs the concentrations were reduced by 90% and after 24 hrs very little of the tropolone could be detected.

The reduction in COMT activity in the striatum was not correlated with the concentration of tropolone. The enzyme activity was reduced to 73% of the normal after 1 hr and showed a similar reduction at 2.5 hrs (table 1). The theoretical reduction in the enzyme activity as calculated from the K_m and K_i values found in the *in vitro* experiments, at the particular tropolone concentration found in the brain at 1 hr was found to be 45% as compared to the actual value of 73%. The explanation for this discrepancy is not known but, possibly some of the tropolone may have been redistributed within the subcellular particles of the homogenate. Brain homogenate, Mg ions and ^{3}H – (methyl)-S-adenosylmethionine was incubated as in an ordinary COMT assay but with tropolone as a substrate instead of DOPAC as usual. No radioactive (methylated) metabolite soluble in chloroform was formed. Neither was it possible to show any metabolite by TLC chromatography of brain extracts. Thus no evidence was found for the presence of an active metabolite of tropolone.

Amines phenolic acids and MAO activity in the striatum

The changes after tropolone in the O-methylated metabolites 3-MT and HVA far exceeded the changes in COMT (tables 2 and 1). HVA was re-

Table 2

Concentration of amines and phenolic acids in rat striatum after tropolone 100 mg/kg intraperitoneally. Values in μg/g wet tissue ± S.E.M. (no. of experiments)

Hours after tropolone	DA	3-MT	DOPAC	HVA
0	3.25 ± 0.245 (6)	0.30 ± 0.058 (6)	1.78 ± 0.146 (7)	0.73 ± 0.107 (7)
0.5			2.24 ± 0.272 (5)*	0.17 ± 0.051 (5)*
1	3.88 ± 0.266 (6)*	0.10 ± 0.039 (6)	2.68 ± 0.202 (5)	0.26 ± 0.058 (5)*
1.5			1.95 ± 0.215 (5)	0.09 ± 0.040 (5)*
2.5	2.66 ± 0.254 (6)	0.11 ± 0.044 (5)	1.87 ± 0.214 (5)	0.26 ± 0.039 (5)
24	3.43 ± 0.313 (6)	0.30 ± 0.062 (5)	2.01 ± 0.153 (5)	0.64 ± 0.077 (5)

Different from value at 0 hours ($P < 0.05$) (Lord's two sample test)

duced to 30% of the control at 0.5 hr and decreased to about 15% at 1.5 hr. The fall in 3-MT closely parallelled that of HVA. The DA concentration showed a small (15%) but significant increase 1 hr after tropolone administration. The changes in DOPAC concentrations followed the DA with a 30% increase at 1 hr, returning to about normal levels at 2.5 hrs. After 24 hrs all the metabolites had returned to control values. At 2.5 hrs a small but significant reduction in MAO activity was found (table 3).

Synthesis of dopamine in the striatum

The half-life of DA in the striatum has been estimated to be about 2.5 hrs (IVERSEN & GLOWINSKI 1966, NEFF *et al.* 1971). Thus, a 20 min. infusion of radioactive tyrosine was considered satisfactory as an index of the DA synthesis rate (NYBÄCK & SEDVALL 1969). It is seen from table 4 that in the non-treated animals the DA and DOPAC fractions showed about the same

Table 3

MAO activity in the striatum after tropolone 100 mg/kg intraperitoneally (mean ± S.E.M. of 4 experiments)

Hours after tropolone	MAO activity (μmol/g/hr)
0	7.9 ± 0.21
1	7.7 ± 0.14
2.5	6.9 ± 0.42*
24	7.1 (2 exp.)

Significantly different from value at 0 hr ($P < 0.05$) (Student t test)

Table 4

Radioactive metabolites in corpus striatum after tropolone, 100 mg/kg intraperitoneally and constant intraperitoneal infusion of ^{14}C-tyrosine over 20 minutes. Values in ‰ of total activity mean of 4 experiments ± S.E.M.

Hours after tropolone	Total activity cpm × 10 ³	DA	3-MT	DOPAC	HVA
0	4.8 ± 1.2	3.5 ± 0.4	0.9 ± 0.1	4.7 ± 0.6	0.5 ± 0.2
1	6.6 ± 2.5	0.5 ± 0.2	0.3 ± 0.1	0.7 ± 0.4	0.1 ± 0.15
2.5	7.7 ± 1.4	0.4 ± 0.1	0.2 ± 0.06*	2.1 ± 0.4*	0.1 ± 0.08
24	8.0 ± 2.2	4.3 ± 1.7	1.3 ± 0.2	4.3 ± 0.5	0.7 ± 0.2

Significantly different from 0 hr
* Significantly different from 24 hrs.
($P < 0.05$) (Lord's two-sample test).

radioactivity after a 20 min. intraperitoneal infusion. Assuming that all the radioactive DOPAC in the striatum originated from radioactive DA, the findings would indicate that the half-life of some DA in the striatum must be in the order of minutes rather than hours. Only a small activity was found in the HVA fraction. After tropolone the activities of all the fractions were significantly reduced returning to normal values after 24 hrs (table 4). For further evaluation of these findings see "Discussion"

Discussion

Murphy *et al.* (1969) found that tropolone is more effective than 4-isopropyltropolone as a COMT inhibitor in the mouse striatum. Goldstein *et al.* (1967) found that 4-isopropyltropolone *in vitro* inhibits tyrosine hydroxylase completely at 10^{-4}M, i.e. a concentration of the same order as the maximal tissue concentrations in the present work. As the DA level in the present experiments rose after tropolone administration, it is likely that this inhibition is not as important *in vivo* and that the observed inhibition of the turnover was a secondary effect due to the increase in catecholamine concentration. Murphy *et al.* (1969) gave DOPA (100 mg/kg) to mice to avoid a possible effect on the tyrosine hydroxylase. They obtained an increase of 100% in the DOPAC concentration in whole brain 1 hr after tropolone (100 mg/kg), while the increase in the present work was 50%. It is possible that tropolone may have some direct effect on tyrosine hydroxylase, but it is not considered significant enough to explain fully the reduction in the rate of synthesis.

The great reduction in methylated compounds in the corpus striatum (3-MT and HVA) as compared to a relatively modest reduction in COMT ac-

tivity when measured in homogenates, may imply that tropolone did not bind very firmly to the enzyme. During homogenization and preparation for the enzyme studies, the tropolone may have been released from the tissue and mixed with the medium. Two and a half hours after the tropolone administration the value of the COMT activity in the homogenates from the striatum was closely related to the theoretical values as calculated from the total concentration of tropolone in the homogenate. By this time the tropolone is diffusing out of the brain and is probably found mostly outside particles in the homogenate. One hour after tropolone there was a discrepancy between the theoretical and the actual enzyme activities. As the COMT determinations were done using crude tissue homogenates in 0.1 M phosphate buffer only 60% of the total enzyme activity in the brain tissue was measured, the remaining being situated inside the particles and hence not available for the substrate (BROCH & FONNUM, unpublished results). It is also possible that a part of the tropolone could be trapped inside the particles after 1 hr.

The increase in DA concentration after tropolone is considered to be due to an inhibition of O-methylation. The DA would be metabolized by the MAO as the O-methylating pathway is blocked, resulting in a consequent increase in DOPAC concentration. As mentioned previously the subsequent reduction in the synthesis rate is throught to be a result of the increase in the amine concentration, probably operating through a negative feedback mechanism acting on the rate limiting synthesizing enzyme, tyrosine hydroxylase (SPECTOR *et al.* 1967). Such a mechanism seems most likely since inhibition of MAO leading to increased amounts of dopamine, has also been shown (COSTA & NEFF 1966) to result in a reduced amine synthesis rate.

The overall half-life of dopamine in the striatum of the rat has been estimated as 2.5 hrs (IVERSEN & GLOWINSKI 1966, NEFF *et al.* 1971). In the present experiments considerable radioactivity had accumulated in the 3-MT fraction after 20 minutes, estimated as about 20% of the activity in the DA fraction. This points to a turnover rate for dopamine in terms of minutes. Moreover the results from the actual chemical analysis of the amines gave a ratio 3-MT/DA of about 1:10 (table 2). These discrepancies can only be explained if one accepts the dopamine storage concept of a "two compartments open in one end" system as suggested for dopaminergic neurons by SHARMAN (1966). At equilibrium the two compartments must possess turnover rates which are inverse to their sizes, and the smaller pool will release the dopamine to the receptor.

The radioactivity of the DOPAC fraction was as high as the activity found in the DA fraction, suggesting a high turnover in the deaminating pathway. It seems therefore necessary to postulate a third pool for DA. This pool is probably very small, consisting of the newly synthesized DA not

incorporated into the synaptic granules. Thus, it seems likely that the metabolic degradation of DA by deamination represents the inactivation of excess DA available for incorporation into the granules, while O-methylation is the main pathway for DA released from the nerve endings. This concept is in agreement with the recent findings by ROFFLER TARLOV *et al.* (1971) which suggest that there may be two different metabolic pathways for dopamine in the striatum of the mouse: one intraneuronal and the other extraneuronal, DOPAC and HVA respectively being the metabolic end products.

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From the Department of Serology and Bacteriology
University of Helsinki, Helsinki, Finland

In Vitro Effect of Anti-Inflammatory Agents on Phagocytosis and Bacterial Killing by Human Neutrophilic Leukocytes

By

Tapani Routu and Timo U Kosunen

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Abstract. The phagocytosis of killed *Staphylococcus epidermidis* by neutrophilic leukocytes was inhibited *in vitro* by indomethacin in a concentration of 5×10^{-4}M, and by hydrocortisone, phenylbutazone, and paracetamol in a concentration of 10^{-3}M. Phagocytosis was slightly stimulated by 10^{-7}M phenylbutazone. Acetylsalicylic acid, mefenamic acid, and phenacetin had no effect. The bactericidal activity of leukocytes against live *Staphylococcus epidermidis* was reduced by 10^{-3}M phenacetin, 5×10^{-4}M mefenamic acid, and 10^{-4}M phenylbutazone and indomethacin, whereas concentrations of 10^{-3}M and above of paracetamol enhanced bacterial killing by leukocytes. Hydrocortisone and acetylsalicylic acid were ineffective. It is probable that concentrations of anti-inflammatory agents sufficient to affect phagocytosis and bacterial killing by neutrophils are as a rule not attained in the organism during treatment, although inhibition may occur *locally* for example *in inflamed tissue, as a* consequence of a low pH, which potentiates the effect of acid anti-inflammatory drugs. This inhibition may be a factor in the mechanism of action of anti-inflammatory compounds.

Key words: Anti-inflammatory agents – leukocytes – neutrophils – phagocytosis.

Enzymes released from the lysosomes of neutrophilic leukocytes, and from the lysosomes of other cells, are regarded as important factors in the inflammatory process. In general, leukocytes do not survive extensive bacterial phagocytosis, but disrupt and die releasing the lysosomal contents into the extracellular space (DOUGLAS 1970) Considerable amounts of lysosomal enzymes can also be released from leukocytes during the phagocytosis of different kinds of particles, without any cellular damage (WEISSMAN *et al* 1969) The inhibition of phagocytosis *could* therefore reduce the inflammatory reaction.

Although a great deal of information is available on the various bio-

logical effects of anti-inflammatory drugs, rather little is known about their effect on phagocytosis and the digestion of bacteria by leukocytes. It has been reported that phenylbutazone interferes with phagocytosis and the intracellular killing of bacteria (ANTWEILER 1957 STRAUSS *et al.* 1968), whereas the results obtained with corticosteroids are contradictory (CREPEA *et al.* 1951 CLAWSON & NERENBERG 1953 HIRSCH & CHURCH 1961 MANDELL *et al.* 1970) The aim of the present paper was to study the effect exerted by some anti-inflammatory compounds on phagocytosis and bacterial killing by human neutrophilic leukocytes *in vitro* Phenacetin and paracetamol were included in this study These are not generally considered to be anti-inflammatory agents, but in some tests they have shown a definite anti-inflammatory activity (DOMENJOZ 1966)

Materials and Methods

Blood donors

The blood donors were young healthy adults, mainly medical students.

Drugs.

The drugs studied were: Hydrocortisone sodium succinate (Organon, Oss), acetyl salicylic acid (Orion, Helsinki), phenylbutazone (Leiras, Turku), indomethacin (Dumex, Copenhagen) mefenamic acid (Parke Davis & Co Hounslow), phenacetin (Orion), and paracetamol (Medipolar Oulu) Phenylbutazone and mefenamic acid were dissolved in 0.1 N-NaOH and indomethacin in 1 N-NaOH, and then neutralized with HCl, as far as possible without precipitation. The other substances were dissolved in saline. The dilutions were made with saline.

Studies on phagocytosis

A brief outline of the methods used for the study of phagocytosis and the bactericidal activity of leukocytes is given below (for details see RUUTU 1972).

Leukocytes. From about 50 ml of heparinized human venous blood, most f the erythrocytes were removed according to the method of BÖYUM (1968) The leukocytes were removed from the plasma by centrifugation, washed with Hanks solution containing 1 per cent bovine serum albumin (HB I), and finally suspended in Hanks solution *containing 12.5 per cent of* autologous plasma.

Bacteria. Staphylococcus epidermidis was grown in broth for 18 hours at 37 The bacteria were killed with formaline, washed three times with saline, and suspended in saline. The suspension was divided into small portions, and stored at −40° One portion was thawed for use in each set of experiments. The same batch was used throughout the study

Phagocytosis assay A volume of 0.8 ml of leukocyte suspension was pipetted into a series of test tubes. The addition of 0.1 ml of the drug solution, or saline in the

control, was made, and the suspension was mixed. If the addition of the drug induced a change in the pH of the suspension, as judged by the colour of the indicator dye, the procedure was modified (also for the controls) in the following way: to 1.4 ml of Hanks solution, 0.2 ml of autologous plasma and 0.2 ml of drug solution were added, after which the pH was neutralized with 0.1 N-HCl or NaOH, and the cells were suspended in 0.9 ml of this solution. This modification to the procedure did not change the results obtained with the controls. After incubation for 30 minutes at 37° 0.1 ml of bacterial suspension was added to the tubes. The final neutrophil concentration was 5 × 10⁶/ml, the ratio of bacteria to neutrophils being about 5:1, and the plasma concentration 10 per cent. The tubes were mixed, left at 37° for a further 6 minutes, and then mixed again. Smears for microscopy were prepared, and stained with Wright's stain.

Two hundred neutrophils were examined from each sample. Counts were made for the phagocytic index, viz. the average number of bacteria phagocytosed by one neutrophil.

Studies on the bactericidal activity of leukocytes

Leukocytes. Leukocytes were prepared in the same way as in the phagocytosis system, but they were finally suspended in Hanks solution containing 0.1 per cent of bovine serum albumin (HB II).

Bacteria. For each day *Staphylococcus epidermidis* was grown in broth for 18 hours at 37° washed three times with saline, and suspended in Hanks solution to a standard optical density.

Assay for bactericidal activity of leukocytes. To 6.4 ml of cell suspension, additions were made of 0.8 ml of pooled human serum and 0.8 ml of the bacterial suspension. The final neutrophil concentration was 1 × 10⁷/ml, and the ratio of bacteria to neutrophils was about 1:1. The suspension was mixed for 5 minutes at 37° on an inclined wheel rotating at 35 r.p.m., 1 ml of the suspension was then pipetted into a series of test tubes, and centrifuged. The supernatant containing the extracellular bacteria was removed carefully. An addition of 0.2 ml of drug solution or saline in the controls was made to 1.8 ml of HB II, and if necessary the pH was corrected with 0.1 N-NaOH or HCl to about 7.4 according to the indicator dye. The cells were suspended in 1.0 ml of this solution: this represented "time zero". After mixing, the tubes were left to stand at 37° for 2 hours, with occasional gentle agitation in order to prevent sedimentation.

For cultures of living bacteria at time zero, and after 1 and 2 hours, 0.1 ml of the suspension was added to 0.9 ml of 1 per cent Triton-X-100: this resulted in complete destruction of the cells. The bacteria retained their viability. Further dilutions were made with distilled water. The bacteria were grown on blood agar slants, and colony counts were made.

Statistical treatment.

Statistical calculations of the significance of the effects of the drugs were based on the t-test. Effects significant at a 0.05 level or below were considered to be significant. (For details, see RUUTU 1972).

Table I

Effect of anti-inflammatory agents on phagocytosis. The number of experiments is indicated in brackets. Mean ± S.E.M. is given.

Drug	Concentration (M)	Phagocytic index, per cent of control
Hydrocortisone	10^{-7} (5)	108.2 ± 5.3
	10^{-6} (5)	107.5 ± 5.7
	3×10^{-4} (4)	105.9 ± 6.9
	10^{-3} (9)	87.3 ± 4.0*
Acetylsalicylic acid	10^{-7} (6)	100.9 ± 3.8
	10^{-6} (4)	97.3 ± 5.3
	10^{-3} (4)	100.1 ± 4.9
Phenylbutazone	10^{-7} (5)	111.4 ± 4.4
	10^{-6} (5)	93.6 ± 3.5
	3×10^{-4} (5)	94.7 ± 7.9
	10^{-3} (7)	78.7 ± 4.0*
Indomethacin	10^{-7} (4)	93.5 ± 5.1
	10^{-6} (5)	93.6 ± 5.5
	10^{-4} (5)	94.1 ± 4.0
	3×10^{-4} (6)	92.1 ± 3.6*
	10^{-3} (7)	63.2 ± 3.6
Mefenamic acid	10^{-7} (5)	101.4 ± 5.0
	10^{-6} (5)	93.7 ± 3.4
	5×10^{-4} (5)	98.3 ± 5.7
Phenacetin	10^{-7} (4)	104.4 ± 3.5
	10^{-6} (5)	108.8 ± 5.7
	10^{-3} (4)	99.1 ± 3.3
Paracetamol	10^{-7} (4)	105.6 ± 6.7
	10^{-6} (4)	100.1 ± 5.3
	3×10^{-4} (4)	101.5 ± 4.1
	10^{-3} (7)	83.5 ± 3.4

* = difference from control significant ($P < 0.05$)

Results

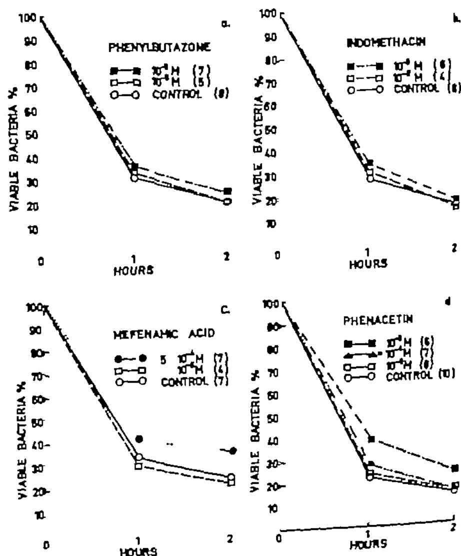
Effect of anti-inflammatory agents on phagocytosis

Table 1 presents the effect of anti-inflammatory agents on phagocytosis. Hydrocortisone in 10^{-3} M concentration slightly inhibited phagocytosis, but had no effect in concentrations of 3×10^{-4} M or less. To some extent, phenylbutazone inhibited phagocytosis at 10^{-3} M. The other concentrations tested did not have any effect, with the exception of 10^{-7} M which produced a slight, but statistically significant, stimulation of phagocytosis.

Indomethacin inhibited phagocytosis in concentrations of 3×10^{-4} and 10^{-3} M. Paracetamol at 10^{-3} M slightly reduced phagocytosis, but not in more dilute concentrations. Acetylsalicylic acid, mefenamic acid and phenacetin did not affect phagocytosis.

None of the tested drugs in the concentrations used caused any detectable light-microscopic morphological changes in the leukocytes, nor did they have any effect on the viability of the leukocytes, measured with the exclusion of 1 per cent trypan blue.

In preliminary experiments, tests were also made on the effects of 10^{-3} M hydrocortisone, phenylbutazone and indomethacin on phagocytosis



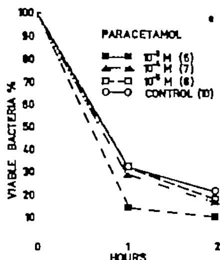


Fig. 1. Effect of anti-inflammatory agents on the intracellular killing of bacteria. The number of experiments is indicated in brackets. The means are given.

in the presence of 80 per cent plasma in the incubation medium instead of 10 per cent. Under these conditions none of the compounds had any effect on phagocytosis.

Effect of anti-inflammatory agents on intracellular bacterial killing

Hydrocortisone and acetylsalicylic acid had no effect on the ability of leukocytes to kill phagocytosed bacteria. Phenylbutazone (fig. 1a) slightly retarded the killing of bacteria in cells in a 10^{-4} M concentration the effect was statistically significant at two hours. No effect was exerted by 10^{-5} M phenylbutazone. Indomethacin (fig. 1b) at 10^{-3} M induced a slight, but constant and statistically significant retardation of bacterial killing, which was visible at one hour but was not observed at two hours. No effect was exerted by 10^{-5} M concentration. 5 × 10^{-4} M mefenamic acid (fig. 1c) clearly interfered with bacterial killing, but 10^{-5} M did not. Phenacetin (fig. 1d) at 10^{-3} M retarded bacterial killing. A similar effect was visible with a 10^{-4} M concentration at one hour but not after two hours. No effect was observed with 10^{-5} M phenacetin. Paracetamol (fig. 1e) behaved differently from the other drugs. In 10^{-5} 10^{-4} and 10^{-3} M concentrations, it enhanced the killing of bacteria in cells to an extent that increased with higher concentrations. With a concentration of 10^{-5} M the effect was first discernible at two hours. On microscopic examination, the leukocytes were broken by Triton-X 100 just as completely when paracetamol was present as in the controls. Even the highest concentration of

paracetamol had no effect on the viability of bacteria, if the bacteria and drug were incubated in HB II without cells. This was also found to be the case with the other drugs in the concentrations used.

Discussion

It has been claimed that cortisone interferes *in vivo* with phagocytosis by leukocytes (CREPEA *et al* 1951), and by the reticuloendothelial system (NICOL *et al.* 1967), whereas in other studies no such effect has been observed (CLAWSON & NERENBERG 1953 HIRSCH & CHURCH 1961). WARD (1966) has shown that hydrocortisone at a high concentration impairs phagocytosis by neutrophils *in vitro* whereas MANDELL *et al.* (1970) could not demonstrate any such effect. Studies concerned with the effect of cortisone on intracellular bacterial killing are also contradictory (CLAWSON & NERENBERG 1953 HIRSCH & CHURCH 1961 MANDELL *et al.* 1970). In this study the highest hydrocortisone concentration used, 10^{-3} M induced slight impairment of phagocytosis, but no effect on intracellular killing was discernible. Probably the inconsistent results are attributable, at least in part, to variations in the experimental conditions.

ANTWEILER (1957) and STRAUSS *et al.* (1968) have shown that phenylbutazone interferes with phagocytosis this has also been demonstrated in this work at the highest concentration used. STRAUSS *et al.* (1968) have furthermore shown that phenylbutazone impairs bacterial killing by leukocytes. A similar trend was observed in this work.

According to ANTWEILER (1957) acetylsalicylic acid inhibits phagocytosis *in vivo* This is contradictory to our results obtained *in vitro*. The difference might be attributable to the hydrolysis of acetylsalicylic acid *in vivo* (WOODBURY 1970) Little is known about the mechanisms by which anti-inflammatory agents influence the functions of phagocytes. Phagocytosis and the intracellular killing of bacteria are complicated processes which may be affected by the drugs in many different ways. The function of phagocytes is dependent on energy-producing processes: phagocytosis on glycolysis, and digestion on oxidative processes (SELVARAJ & SBARRA 1966). It has been demonstrated that non-steroid anti-inflammatory agents uncouple oxidative phosphorylation (WHITEHOUSE 1968). STRAUSS *et al.* (1968) have shown that phenylbutazone in a concentration of 5×10^{-3} *M* inhibits both glycolysis and the flow of glucose through the hexose monophosphate pathway The latter process is intimately involved in the digestion phase. They also showed that phenylbutazone inhibits the production of H_2O_2, which is an important factor in the bactericidal systems of neutrophils. MCCURRACH *et al* (1970) have demonstrated that phenylbutazone inhibits

the oxygen uptake of human neutrophils in concentrations of 10^{-4} M or more.

Membranes play an important role in both phagocytosis (formation of phagosome), and in intracellular digestion (fusion of phagosome and lysosome) Many anti-inflammatory compounds are membrane-active, so that they can stabilize or labilize the erythrocyte membrane, depending on their concentration (INGLOT & WOLNA 1968), and stabilize lysosomes (WEINER & PILIERO 1970) According to WHITEHOUSE (1965), the action of anti-inflammatory agents on membranes may be considered as their most important mechanism of action, and HOUCK (1969) has stated that drug control of the acute inflammatory response may profitably be attempted at the levels of stabilizing membranes of lysosomes and white blood cells.

Hydrocortisone (KETCHEL *et al.* 1958) and indomethacin (PHELPS & McCARTHY 1967) have been shown to inhibit leukocyte migration, and hydrocortisone to inhibit the chemotactic response (WARD 1966). The inhibition of leukocyte migration might be one factor involved in the present results, but in suspension, mixed at intervals, the active migration of leukocytes would be expected to play no important role.

As a rule, in this study the concentrations of drugs found to affect the function of phagocytes were relatively high. However many anti-inflammatory agents are given to patients in rather high amounts. The therapeutic plasma concentration of salicylates may be as high as $2\text{–}3 \times 10^{-3}$ M (WOODBURY 1970), and that of phenylbutazone about 5×10^{-4} M (CATANESE *et al.* 1969), whereas indomethacin (CATANESE *et al* 1969), and mefenamic acid (GLAZKO 1966) in therapeutic doses produce lower plasma concentrations, about 2×10^{-6} M and 5×10^{-5} M respectively Binding to proteins reduces the concentration of free active drug. In these experiments, hydrocortisone, phenylbutazone and indomethacin in 10^{-3} M concentration, obviously for this reason, did not affect phagocytosis in the presence of 80 per cent plasma in the incubation medium, although an effect was discernible in Hanks solution containing 10 per cent plasma. In plasma, phenylbutazone is bound to proteins to an extent exceeding 90 per cent (WOODBURY 1970), hydrocortisone (KONO *et al.* 1966) and indomethacin (WOODBURY 1970) to about 90 per cent, and the other drugs studied to a lesser degree (WOODBURY 1970; GLAZKO 1966) In this study 10 per cent *plasma was present in most phagocytosis experiments, but none in the* bactericidal tests.

The distribution of drugs in the organism can be uneven, as is known in regard to hydrocortisone (SAMUELS 1966), and phenacetin (SMITH 1958). Paracetamol is markedly concentrated in the renal medulla (BLUHMLE & GOLDBERG 1967). Phenylbutazone may be concentrated in inflammatory sites (WILHELMI & PULVER 1955)

The pH is abnormally low in the inflammatory area. Tissue injury can induce a fall in pH to 6 8–6 0 within some minutes (INGLOT & WOLNA 1968), and the pH may fall far below 6 (WHITEHOUSE 1965) This is likely to increase the concentration of acid anti-inflammatory agents in the cells, and potentiate their anti-inflammatory activity (INGLOT & WOLNA 1968 WHITEHOUSE 1965)

If the anti-inflammatory agents essentially disturb the function of phagocytes *in vivo* this could lead to a reduced resistance to infection. Ample evidence exists of the increased susceptibility to infections caused by corticoids (cf. FAUVE & PIERCE-CHASE 1967) but very little of similar actions as far as the other anti-inflammatory drugs are concerned. It has been shown that phenylbutazone (AGARWAL 1967) and indomethacin (JACOBS 1967) aggravate infections in experimental animals, and that they are suspected of reducing the resistance of some patients to infection (HANDFIELD 1955 SOLOMON 1966) It has been suspected that an increased frequency of urinary infections occurs in connection with analgesic nephropathy induced by phenacetin, together with other compounds (SHELLEY 1967), but these infections are obviously secondary complications of the later phases of the nephropathic process, and do not form an essential part of it (LEVIN 1969) It is thus evident that anti inflammatory agents, with the exception of corticoids, do not essentially increase the susceptibility to infections, and that corticoids reduce resistance to infection, at least mainly by means other than by interference with the function of the phagocytes.

One interesting point is that phenacetin, together with paracetamol, which is the major metabolite of the former (WOODBURY 1970), had effects which were considerably different. Phenacetin did not have any influence on phagocytosis, and retarded the killing of bacteria in the cells, whereas paracetamol slightly inhibited phagocytosis, and clearly enhanced the killing of bacteria. There is no obvious explanation for this at present. The therapeutic plasma concentrations of paracetamol may rise to about 7×10^{-4} M (SMITH 1958). Concentrations that still enhanced intracellular bacterial killing in these experiments can obviously be found in the organism, particularly since it is known that paracetamol is bound to proteins to a rather low degree (WOODBURY 1970). The drug may also be concentrated in the renal medulla sufficiently to attain concentrations capable of inhibiting phagocytosis.

It seems that concentrations of anti-inflammatory drugs sufficient to affect phagocytosis and bacterial killing by neutrophils are not generally attained in the organism during treatment. However the uneven distribution of the drugs, and the low pH in inflamed tissue which probably potentiates the effect of acid anti inflammatory agents, may produce conditions locally

in which anti-inflammatory drugs inhibit the function of phagocytes. This inhibition could be a factor concerned in the mechanism of action of anti-inflammatory drugs.

Acknowledgements

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From the Institute of Pharmacology and Toxicology The Royal Veterinary and Agricultural University Bülowsvej 13, DK 1870 Copenhagen Denmark

Toxicity of Polychlorinated Biphenyls (PCB) to Goldfish *

By

Marja Liisa Hattula and Ole Karlog

(Received December 9 1971 Accepted December 22, 1971)

Key-words. PCB – toxicity – goldfish.

Due to strong adsorption of PCB's from water solutions to glass surfaces (HATTULA & KARLOG 1971) the evaluation of their toxicity to fish based on experiments performed in glass aquaria may be unreliable. For Aroclor 1260 SCHOETTGER (cited from PEAKALL & LINCER 1970) found that the 96 hrs TLm using cutthroat trout was 60.9 mg/l while the figure for the more soluble Aroclor 1221 was 1.2 mg/l. Since PCB in experiments with Clophen A 50 has not been found to be adsorbed to aluminium-foil surfaces (HATTULA & KARLOG 1971) the following investigation was performed using veil-tailed goldfish, with an average weight of 1.9 g. Aluminium-foil containers holding 6 l of water served as aquaria for groups each of 19 fishes. Clophen A 50 (Bayer) was used and diluted from a stock solution containing 10 mg PCB per ml in acetone. The concentrations of PCB in water were 0 0.5 1.5 2.0 2.5 and 4 0 p p.m and the concentration of acetone was adjusted to 0.5 ml/l in all the containers. The containers were replaced every day and the fish fed half an hour before being moved to a new aquarium filled with freshly prepared solution.

Dry plant mixture (Tetra Phyll) with a background contamination of PCB of 0.2 p p.m. was used as feed in an amount of 1 g per day to 19 fishes. Aeration of the water was arranged by air pressure. The temperature during the experiment was between 21°–23

Fish were removed when found dead, rinsed by dipping in distilled water and then homogenized. PCB analyses were performed by the method of AHLING & JENSEN (1970) with slight modifications.

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Present address Department of Organic Chemistry University of Jyväskylä, Jyväskylä, Finland.

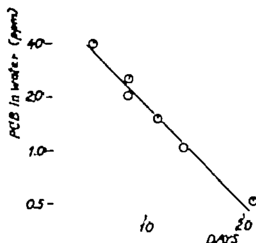


Fig. 1 Time of 50 per cent mortality as a function of concentration of PCB in water (in log scal).

Table 1

Time of appearance of symptoms in days from the starting point.

PCB in water (p.p.m.)	0	0.5	1.0	1.5	2.0	2.5	4.0
Loss of appetite	–	12	6	4	4	4	2
Changes in colour	–	9	7	6	6	5	3
Mild incoordination	–	15	8	5	5	4	3
Severe incoordination		22	9	7	7	5	4

Table 2

Background contamination and lethal concentrations of PCB in whole specimens.

PCB in water (p p.m.)	0	0.5	1.0	1.5	2.0	2.5	4.0
PCB in fresh tissue (p p.m.)	0.21	250	253	271	.56	293	324
	(0.12–0.30)	(122–471)	(171 390)	(135–428)	(179–321)	(176–473)	(217–623)

One of the fishes from the control group died during the experiment which lasted for 31 days. The time at which 50 per cent of the fish were killed is shown in fig. 1

The harmful effects of PCB could easily be observed (table 1) The fish lost their appetite and their bright orange colour turned to pale yellow the changes being most obvious with the highest concentrations of PCB. The effect on the nervous system could be observed as incoordinated movements after which the fish turned to side positions. To ensure that the effects were caused by PCB and not by starvation, another control group of 16 fishes were starved in clean water for 10 days. With the exception of a 25 per cent loss of weight and two deaths no abnormalities were noted.

Table 2 shows the concentrations of PCB absorbed by the fish during the experiment and also the background contamination. There were large individual variations, but the differences in PCB concentrations between the experimental groups are not significant. Due to the very low fat content of the fish (0.34–1.14 per cent) the calculated PCB concentrations in the fat of the dead fish are remarkably high (13.5 mg/g to 93 1 mg/g) or a mean of 35 6 mg/g fat for all the experimental groups.

When a fifty per cent chlorinated PCB is kept in solution and is not adsorbed to the surfaces of the aquaria, lethal water concentration to goldfish are almost of the same order as found for a more soluble component in experiments using trout (vide PEAKALL & LINCER 1970). The change in colour of the fish observed during the experiment might be due to disturbances in fatty acid metabolism (WALLACH 1971), and might decrease resistance to fungal infections of the body (HANSEN *et al* 1971) The concentrations of PCB in dead fishes observed in this experiment are 250–325 p.p.m. in whole specimens and are comparable to for instance the contents in the liver of Bengales finches – 345 p p.m – of 50 per cent mortality (PRESTT *et al* 1970).

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From the Department of Pharmacology, Royal Veterinary College,
S-10405 Stockholm 50, Sweden

Responses of the Rabbit and Cat Urinary Bladders *In Situ* to Drugs and to Nerve Stimulation

By

Sven Erik Sjöstrand, Christer Sjögren and Carl G. Schmiterlöw

(Received November 24, 1971. Accepted January 28, 1972)

Abstract. The response to atropine, hexamethonium, nicotine, emepronium bromide and some adrenergic compounds has been studied on the urinary bladder of the rabbit and the cat. It was shown that atropine caused a very small or no reduction in the response to electrically stimulated parasympathetic nerves in the urinary bladder of the rabbit. In the cat a dose dependent blockade was obtained after atropine administration. The stimulating response was abolished by hexamethonium or nicotine, suggesting that there are nicotinic receptors in the parasympathetic pathway. It is possible that the parasympathetic nerves running to the rabbit urinary bladder end in such nicotinic receptors on the effector cells in the detrusor muscle. Emepronium bromide, a potent anticholinergic compound, used clinically as urological drug, was found to block the stimulatory response, which was interpreted as an effect on parasympathetic nicotinic receptors. Experiments with isoprenaline, adrenaline and noradrenaline showed that there are adrenergic α- and β-receptors in the urinary bladder of the rabbit.

Key-words: Urinary bladder – nerve stimulation – anticholinergic effects – catecholamine effects.

In the search for anticholinergic drugs acting on the urinary bladder we have found that the bladder of the rabbit responds to atropine in a different way from that of the cat. Very little is known about the effect on the rabbit urinary bladder of compounds acting on the autonomic system. In other species, such as the cat, dog, and guinea-pig, numerous investigations have been undertaken and the results thus obtained are interpreted in different ways. Anyone studying the effect of atropine on pelvic nerve stimulated bladders has found that the stimulatory response is only partially reduced by this compound. This observation has led to much speculation and some investigators believe that this is a non-cholinergic link in the parasympathetic pathway (Henderson & Roepke 1934, Ambache & Zar 1970). Carpenter & Rand (1965) postulate that the myoneural junctions of the bladder

are not accessible to atropine, an assumption which is not accepted by DUMSDAY (1971).

In the present study the effect of some compounds acting on the autonomic nervous system on the response to electrically stimulated parasympathetic nerves running to the bladder as well as on the basic tone of the bladder has been investigated.

Material and Methods

Animals and anaesthesia.

Eighty-six rabbits and ten cats were used. The anaesthetized animals were placed in a supine position. The abdomen was opened by a midline incision. One of the vertebral joints between Th 12 and L2 was dissected free. The disc was partly removed and the spinal cord was cut.

The rabbits (2.0–2.5 kg body weight) were anaesthetized by intravenous injection of a 20 % urethane solution. The dose was 1.4 g urethane/kg. The cats (3–4 kg) were anaesthetized by intraperitoneal injection of pentobarbitone (mebumalum NFN, mebumalnatrium® ACO). The dose was 35 mg/kg.

Intravesical pressure recording

Both ureters were ligated rather close to the kidneys. A polythene tubing (Portex PP 90) was inserted into the urinary bladder through a slit either in the urethra or in the detrusor muscle between the neck and base of the bladder. The tubing was tied with a piece of silk thread to avoid leakage. A transducer for recording the intravesical pressure (Statham P 23 AC) was connected to the tubing. The system was filled with physiological saline and the bladder was rinsed a couple of times with saline and finally filled with 10–20 ml saline. The intravesical pressure was recorded on a Grass ink writing oscillograph. One hour was allowed to elapse before recordings were made.

A polythene tubing (Portex PP 20) was inserted either into one of the femoral veins or into the abdominal aorta. All injections were made through either of these catheters.

Technique for nerve stimulation.

A bipolar platinum electrode was placed on the parasympathetic pelvic nerves on one side of the bladder as they run along the blood vessels immediately before they enter the bladder tissue. Stimulatory currents were supplied by a Grass model S4 stimulator. The stimulation consisted of square wave pulses of 0.5–5 msec. duration and an intensity of 5–10 V. Stimulation was given in the form of bursts of such pulses. The duration of the individual bursts was 5 sec., and the frequency within the bursts was varied between 0.5 and 25 pulses/sec.

Drug administration.

The urinary bladder of three anaesthetized rabbits was cannulated as described above, but no stimulation was applied. Some adrenergic drugs were injected intravenously and their effect on the normal intravesical pressure (basic tone) was recorded. Control injections with physiological saline were made.

Table 1

Compounds, doses, animal species and number of experiments.

Compound	Dose mg/kg	Animal species	No. of experiments
Atropine (atropine sulphate, ACO)	0.5 –12	rabbit, cat	31,3
Emepronium bromide (cetiprin® Recip)	0.5 – 4	rabbit, cat	34 4
Nicotine (nicotine sulphate)	0.01– 1.2	rabbit	5
Hexamethonium (hexamethonium bromide)	0.5 –20	rabbit, cat	10,1
Dihydroergotamine (orstanorm® Sandoz)	1	rabbit, cat	10,1
Succinylcholine (succinylcholine iodide)	0.5 – 1.2	rabbit, cat	6,2
d-tubocurarine	0.1	rabbit, cat	6,2
Adrenaline (base)	0 010*	rabbit	3
Noradrenaline (base)	0 010*	rabbit	3
Isoprenaline (isoprenaline sulphate)	0.005*	rabbit	3
Phentolamine (regitin® CIBA)	2	rabbit	3
Propranolol (Inderal® ICI)	1	rabbit	3

Amount injected independent of body weight.

Table 1 shows the compounds, doses and animals used as well as the number of experiments with each compound.

Results

The response to nerve stimulation was recorded as a rise in the intravesical pressure. There were no apparent differences in the normal response between rabbits and cats. Control injections of physiological saline in volumes used for the test compounds did not change either the stimulatory response or the basic tone.

In some experiments the stimulation frequency was varied in order to

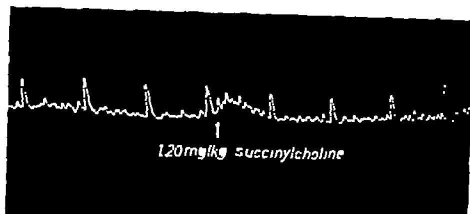


Fig. 1. *Rabbit*. Urethane anaesthesia. The effect of 1.2 mg/kg succinylcholine administered intravenously on the response to electrical nerve stimulation of the urinary bladder.

out when a maximum or minimum response was obtained. At frequencies below 2 imp./sec., no effect at all was recorded during nerve stimulation. With increasing frequency the response increased and reached a maximum at 10 imp./sec. In the following experiments recordings were made at both 2 and 10 imp./sec.

Dihydroergotamine.

In order to check whether adrenergic as well as cholinergic fibres were stimulated and responsible for the increase of the intravesical pressure, an α-receptor blocking compound was given. Dihydroergotamine was injected intravenously into several animals. No effect whatsoever on the response to nerve stimulation was observed. However, this compound caused a long-lasting increase in the basic tone.

Succinylcholine

Succinylcholine (suxamethonium NFN) and d-tubocurarine were injected into some of the animals in order to find out whether neuromuscular junctions were involved. The results showed that these compounds had no effect on the electrically induced increase in the intravesical pressure (fig. 1). In a few experiments a high dose of succinylcholine resulted in a transient increase in the basic tone.

Atropine

After intravenous injection of atropine into *rabbits* very little or no reduction of the stimulatory response was observed (fig. 2). In some animals,

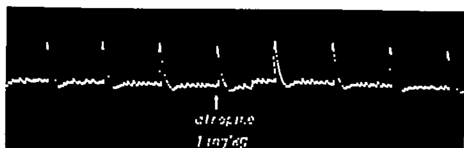


Fig. 2. *Rabbit.* Urethane anaesthesia. The effect of 1 mg/kg atropine administered intravenously on the responce to electrical nerve stimulation of the urinary bladder

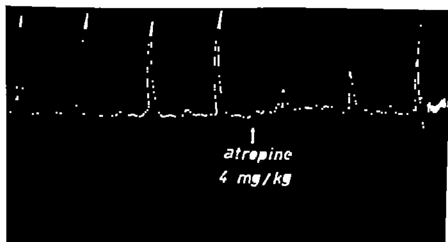


Fig. 3. *Cat.* Pentobarbitone anaesthesia. The effect of 4 mg/kg atropine administered intra-arterially on the response to electrical nerve stimulation of the urinary bladder

atropine was injected into the aorta in doses of up to 12 mg/kg. In spite of the high dose, only a small reduction of the induced increase of the intravesical pressure was found. The reduction never exceeded 30 / of the initial value.

In several rabbits, a rise in the basic tone was seen when a high dose of atropine was given. This effect lasted for 2–5 minutes.

In the *cat* intra-arterially administered atropine caused a pronounced block of the stimulatory response (fig. 3). However complete block never occurred even if the dose was increased to 4 mg/kg. The reduction was esti mated to be about 80–90 / when the highest dose was given. In the cat experiments, atropine injection was also followed by a slight rise in the base line.

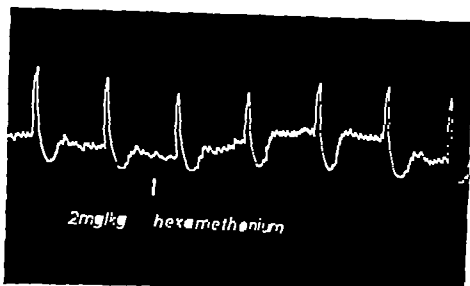


Fig. 4 *Rabbit* Urethane anaesthesia. The effect of 2 mg/kg hexamethonium administered intravenously on the response to electrical nerve stimulation of the urinary bladder

Hexamethonium.

After intravenous injection of hexamethonium into *rabbits* the electrically induced increase in the intravesical pressure was reduced. At 3 mg/kg, the amplitude was reduced to about 50 / of the initial value. The blocking ef fect of a single dose lasted for more than 30 minutes. After repeated injec tion of hexamethonium, the pressure amplitude gradually diminished and finally no response to nerve stimulation was obtained.

The base line was also changed, following hexamethonium administration (fig. 4). Like emepronium bromide (see below), it caused an increase in the basic tone, which was more apparent than that after the emepronium bromide injection. When 3 mg/kg or higher doses were given, the basic tone never completely returned to the preinjection level.

In one *cat* 0.5 mg/kg hexamethonium was injected. The results showed a marked, but not total blockade of the stimulatory response.

Nicotine

Nicotine injected intravenously in doses below 0.1 mg/kg, did not cause any changes in the stimulatory response or basic tone in the bladder of the rabbits. When larger amounts of nicotine were given, a sudden increase in the intravesical pressure was observed immediately after the administration. This drug-induced effect increased with the dose and became rather marked at 1.2 mg/kg (fig. 5). Apart from this stimulatory effect of nicotine, it was

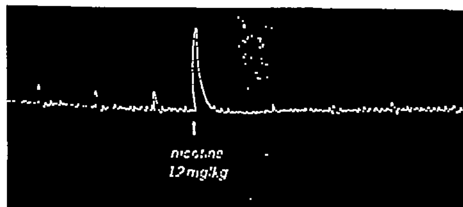


Fig. 5. *Rabbit*. Urethane anaesthesia. The effect of 1.2 mg/kg nicotine administered intravenously on the response to electrical nerve stimulation of the urinary bladder

observed that this compound caused a dose dependent blockade of the response to nerve stimulation. After repeated injections of nicotine, the blockade gradually became complete.

Emepronium bromide

In the *rabbit* this compound was found to cause a reduction in the stimulatory response. After an intravenous injection of 0.5 mg/kg a small reduction in the amplitude was observed. The blocking effect became more marked with increasing doses. At a single dose of 1 mg/kg the stimulatory response was reduced to about 50 / and at 4 mg/kg the reduction was 90–95 / of the initial response (fig. 6) The effect was maximal immediately after the injection. The stimulatory response then gradually increased and reached a steady level when the amplitude was about 75 / of that recorded before the injection of the compound. When emepronium bromide was given repeatedly to one and the same animal, the steady level of the amplitude gradually became lower and finally no response at all could be detected on nerve stimulation.

In several experiments, the injection of emepronium bromide was followed by a rise in the basic tone, starting immediately after the injection. The maximum effect was reached 2–3 minutes after the administration of the compound. The base line never completely returned to the initial level, but remained slightly elevated throughout the experiment.

In the *cat* 0.5 mg/kg emepronium bromide caused a 75 / reduction of the response to nerve stimulation. At 4 mg/kg a complete blockade of the response lasting for about 10 minutes was observed. It was also observed that the spontaneous activity in the bladder which normally is rather promi-

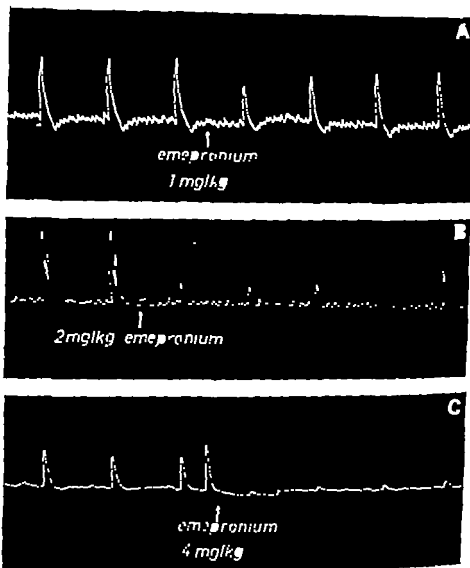


Fig. 6. Rabbit. Urethane anaesthesia. The effect of 1–4 mg/kg emepronium bromide administered intravenously on the response to electrical nerve-stimulation of the urinary bladder

nent in the cat was reduced to a minimum, even after the smallest dose of emepronium bromide.

Adrenergic compounds

The effects of some adrenergic compounds on the basic tone were also studied in 3 *rabbits*

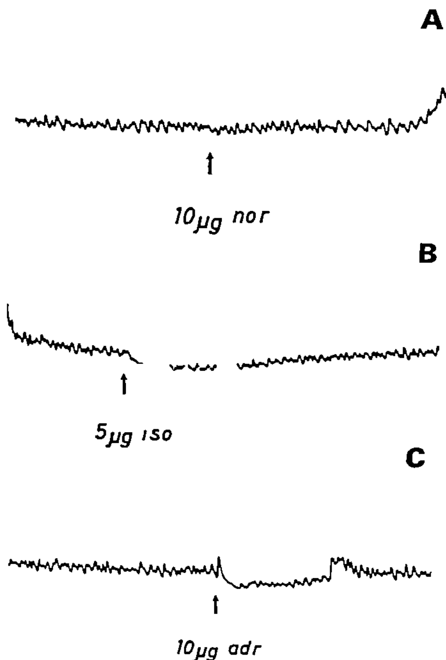
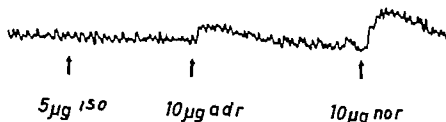


Fig. 7 *Rabbit*. Urethane anaesthesia. The effect of 10 µg noradrenaline (A), 5 µg isoprenaline (B) and 10 µg adrenaline (C) on the basic tone of the urinary bladder



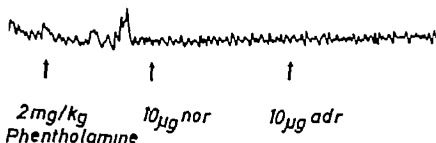


Fig. 8. *Rabbit* Urethane anaesthesia. A. The effect of 5 μg isoprenaline, 10 μg adrenaline and 10 μg noradrenaline on the basic tone of the urinary bladder following premedication with propranolol. B The effect of 10 μg noradrenaline and 10 μg adrenaline on the basic tone following premedication with both propranolol and phentolamine.

As seen in fig. 7A, injection of noradrenaline was without effect. However the administrations of isoprenaline and adrenaline were followed by a reduction in the basic tone (fig. 7B and C). After premedication with propranolol, a β-receptor blocking compound, the effect of isoprenaline was completely abolished. Injections of adrenaline and noradrenaline after β-receptor blockade were followed by a temporary rise in tone (fig. 8A). When the animals were premedicated with both α- and β-receptor blocking compounds (phentolamine and propranolol), the effects of noradrenaline and adrenaline were completely blocked (fig. 8B)

Discussion

It is well known that the urinary bladder of cats and dogs has a dual innervation. There are cholinergic as well as adrenergic nerves and they are believed to play a physiological role in micturition.

When cholinergic sites are experimentally stimulated either by nerve stimulation or chemically the detrusor muscle contracts, resulting in an increase in the intravesical pressure. In the present investigation cholinergic nerves running to the urinary bladder were electrically stimulated. The effect of adrenergic α-receptor blockade on the shape of the stimulus response curve of the urinary bladder does not suggest an involvement of the adrenergic fibres in the contractions produced. The stimulating characteristics rather resemble those of the parasympathetic fibres of the isolated colon and ileum of the rabbit (GARRY & GILLESPIE 1955 DAY & RAND 1961)

Succinylcholine and d-tubocurarine did not affect the stimulatory response indicating a transmission mechanism different from that in striated muscles.

Atropine is well known to cause blockade of post-ganglionic cholinergic sites. However in the present rabbit experiments, atropine seemed to be a rather poor blocker of the response to cholinergic nerve stimulation. The result was the same whether atropine was given intravenously or intra-arterially in high doses.

In certain strains of rabbits an enzyme, atropine esterase has been isolated in the serum, which is said to reduce the atropine response by inactivation. In the present experiments a high dose of atropine was given *intra-arterially* just before the artery reaches the urinary bladder in order to get a high concentration of the substance in the effector organ. The blockade of the stimulatory response was poor in spite of the high dose suggesting that the poor effect of atropine is due to factors other than to inactivation by esterases. It should also be pointed out that according to some authors (AMBACHE 1955 AMBACHE & LIPPOLD 1949 BLASCHKO 1962 HOBBIGER & LESSIN 1955 SAWIN & GLICK 1943) many strains of rabbits seem to lack this enzyme.

A partial blockade of a pelvic nerve stimulatory response by atropine has been observed in other species (LANGLEY & ANDERSON 1895 HENDERSON & ROEPKE 1934 AMBACHE & ZAR 1970). EDGE (1955) reported that atropine administration actually potentiated the response to pelvic nerve stimulation in cats. This was, however not confirmed in the present experiments. Intra-arterially injected atropine caused a dose dependent reduction of the response to cholinergic nerve stimulation in the cat. This indicates that there must be atropine sensitive mechanisms in the urinary bladder of the cat, which can be activated by nerve stimulation. In this respect there is an obvious difference in the response to atropine between rabbits and cats.

Following hexamethonium injections, the stimulatory response gradually diminished and finally no response to nerve stimulation was obtained. As hexamethonium is a well known antagonist of nicotinic receptors in ganglia, the present results indicate that there are receptors in the urinary bladder, presumably nicotinic ones, which are activated by cholinergic nerve stimulation. A similar response to hexamethonium has been observed in cats (EDGE 1955 EDVARDSEN 1967). In rats the effect of pelvic nerve stimulation is not influenced by ganglion blocking drugs (VANOV 1965).

That nicotinic receptors are involved is confirmed in the experiments, in which nicotine was injected. The results showed that nicotine initially caused an increase of the intravesical pressure similar to that produced by nerve stimulation. Repeated injections of nicotine caused a complete blockade of the stimulatory response similar to that of hexamethonium.

The results suggest that there are nicotinic receptors present in the stimulatory pathway but very few muscarinic receptors. It is possible that the parasympathetic nerve fibres end in the nicotinic receptors on the urinary bladder of the rabbit. In the cat, both types of receptors seem to be present, and the nicotinic receptors are probably localized in conventional ganglionic structures.

Emepronium bromide is used clinically as a urological drug in the treatment of urinary incontinence (JÖNSSON & ZEDERFELDT 1957 BROCKLEHURST *et al.* 1969) Emepronium bromide is a potent anticholinergic compound with sympathetic ganglion blocking properties. The anticholinergic effect has been tested on the isolated guinea-pig intestine and the ganglion blocking effect has been tested on the stimulated nictitating membrane of the cat (SJÖSTRAND 1968). It is chemically defined as a quaternary ammonium compound (HANSSON & SCHMITERLÖW 1961) In the present study it was found that this compound caused a marked blockade of the electrically induced stimulatory effect on the rabbit urinary bladder The difference in effect between emepronium bromide and atropine suggests that the former compound blocks nicotinic receptors in the parasympathetic pathway to the urinary bladder

The existence of adrenergic receptors in the urinary bladder of cats and dogs has previously been demonstrated (BOYARSKY *et al.* 1968 EDVARDSEN 1967 EDVARDSEN & SETEKLEIV 1968 DHASMANA *et al.* 1970). In the present experiments injection of isoprenaline and adrenaline caused a decrease in the intravesical pressure of the rabbit, as shown by the lowering of the basic tone. This effect was abolished after adrenergic β-receptor blockade, indicating that there are β-receptors present in the urinary bladder of the rabbit. The β-receptor activity in the rabbit urinary bladder seems to be the same as found in the bladder of other species (see ref. above).

The increase in the intravesical pressure following injection of adrenaline

and noradrenaline after β-receptor blockade was abolished by phentolamine. These results indicate the presence of α-receptors in the urinary bladder of the rabbit, similar to those found in other species (see ref. above) The present results are in agreement with those previously obtained in experiments *in vitro* with muscle strips from the rabbit urinary bladder (EDVARDSEN & SETEKLEIV 1968)

The rise in the basic tone observed after the injection of hexamethonium, emepronium bromide high doses of atropine succinylcholine and d tubocurarine is interpreted as a blockade of the transmission in the intact adrenergic nervous supply to the urinary bladder All these substances are known to block at sympathetic ganglion sites. According to EDVARDSEN (1967) β-receptors are activated during the collecting of urine causing a reduction in the intravesical pressure and since β-receptor activation of the rabbit urinary bladder is followed by a lowering of the basic tone, it is believed that when this sympathetic effect is blocked at the ganglion level, there is an increase in the basic tone. This may be a possible explanation for the results obtained.

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From the Department of Pharmacology AB Bofors, Nobel Pharma, S-431 33 Mölndal, Sweden, and the Department of Zoophysiology University of Göteborg, Sweden

Mechanical and Electrophysiological Effects of Some Local Anaesthetic Agents and Their Isomers on the Rat Portal Vein

By

G. Åberg and B. Wahlström

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Abstract: Mechanical and electrophysiological effects of the DL, L and D-forms of mepivacaine and bupivacaine and of the optically inactive compound tetracaine have been studied on the isolated rat portal vein. After the addition of the local anaesthetics to vessels in normal Krebs solution there was an initially increased spike activity accompanied by increased mechanical activity. L(+)-mepivacaine was more potent than D(−)-mepivacaine in increasing the tension, but there was no significant difference between L(−)- and D(+)-bupivacaine. After the initial contractile phase the tension decreased and disappeared despite continuous spike activity. The relaxation appeared and proceeded faster with high than with low concentrations of local anaesthetics; the relaxations also proceeded faster in experiments with D(−)-mepivacaine than with L(+)-mepivacaine. At a later stage the spike discharge was also blocked. In muscles that were contracted with K or noradrenaline the local anaesthetics caused relaxation. D(−)-mepivacaine was more potent than L(+)-mepivacaine, but there was still no difference between L(−)- and D(+)-bupivacaine.

Key-words: Local anaesthetics – optical isomers – vascular effects.

Local anaesthetic agents have since long been known to relax smooth muscle: some agents such as cocaine also have a contractile action (ROTH 1917). It has been demonstrated that mepivacaine (carbocaine® Bofors, Nobel-Pharma, Sweden) has a vasoconstrictor action *in vivo* (POITTO & SCHEININ 1958; DU MESNIL DE ROCHEMONT & HENSEL 1960; ADLER *et al.* 1969; ÅBERG & ADLER 1970) and there is also a difference between the vascular effects of the stereoisomers of mepivacaine in man: the L(+)-isomer has a more pronounced vasoconstrictor action than the D(−)-isomer (ÅBERG & ADLER 1970).

In order to get some insight into the mode of action of the vascular effects of local anaesthetics and their isomers, we have studied the action of mepivacaine and bupivacaine (marcaine® Bofors, Nobel-Pharma, Sweden) which have optically active isomers and also of tetracaine on the mechanical and electrical response of the isolated rat portal vein.

Material and Methods

Preparations of portal veins were obtained from male Sprague Dawley rats, weighing 180–210 g. The animals were killed by stunning and bleeding. When only mechanical activity was recorded the vessel was mounted in an organ bath with a volume of 40 ml. The vessel was connected to an isometric tension transducer (*Grass FT 03B*) which was connected to a Grass polygraph. When both the electrical and the mechanical activities of the muscle were recorded simultaneously the sucrose-gap technic was used (STÄMPFLI 1954).

The muscle showed rhythmic contractions and relaxations. In order to measure quantitatively the effect of the local anaesthetic agents on the myogenic activity the tension was measured at prefixed times and changes expressed in per cent of the maximal tension of the muscle during spontaneous activity. The "normal" Krebs solution contained (mM): Na^+ 137.47; Ca^{2+} 2.49; K^+ 5.94; Mg^{2+} 1.19; Cl^- 134.11; HCO_3^- 15.48; $H_2PO_4^-$ 1.19 and glucose 11.5. The "K^+-high" Krebs solution contained 128 mM K^+ and was obtained by replacing NaCl of the normal solution with KCl. The solutions were kept at 37° and were aerated with a gas mixture containing 97 % O_2 and 5 % CO_2. The local anaesthetics used in this investigation were DL-mepivacaine (M = 283), L(+)-mepivacaine, D(−)-mepivacaine, DL-bupivacaine (M = 325), L(−)-bupivacaine, D(+)-bupivacaine and tetracaine (M = 301).

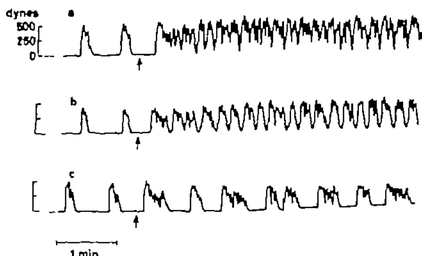


Fig. 1. Immediate mechanical effects on the isolated rat portal vein of
a) L(+)-mepivacaine 6 × 10^{-4} g/ml,
b) DL-mepivacaine 6 × 10^{-4} g/ml
c) D(−)-mepivacaine 6 × 10^{-4} g/ml.

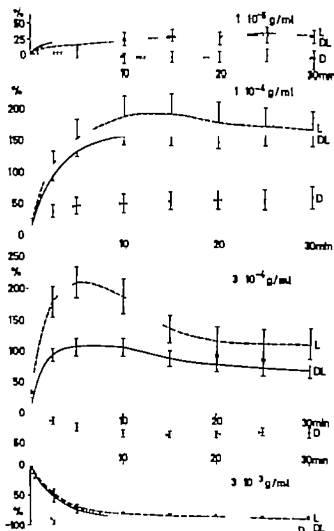


Fig. 2. Diagram showing the effects of DL- L(+)- and D(−)-mepivacaine on the tension in the rat portal vein. Changes of the tension are expressed as per cent of the maximal tension of the individual muscles during spontaneous activity. Each curve shows mean ± S.E.M. from 6–8 experiments.

Results

Mechanical effects of mepivacaine.

A direct registration of an experiment with DL-mepivacaine and its isomers in a concentration of 6×10^{-4} g/ml is shown in fig. 1

In fig. 2 the mean time-response relationships at different concentrations

of mepivacaine are shown. In a final concentration of 1×10^{-5} g/ml both DL- and L(+)-mepivacaine significantly increased the tension during at least 30 min., whereas the D(−)-isomer had no effect in this concentration. In a *ten times higher* concentration, the increasing effect on the tension of DL- and L(+)-mepivacaine was still more marked and even the D(−)-isomer had a moderate contractile effect.

In a concentration of 3×10^{-4} g/ml a relaxing action of the compounds began to appear. This was most marked with the D(−)-isomer which reduced the tension by about 40 per cent. In tests with the L(+)-isomer and the racemate the increase in tension was reduced after 5–15 min.

In a still higher concentration (3×10^{-3} g/ml) only a relaxing action of the compounds was evident, the D(−)-isomer completely relaxed the vessel.

Mepivacaine in a low concentration thus had a contractile action on the rat portal vein. In a higher concentration, the compound had instead a marked relaxing action. There was a quantitative difference between the isomers: the L(+)-isomer increased the tension over a wider range of concentrations whereas the relaxing action of the D(−)-isomer was more marked. The action of the racemate was mainly like that of the L(+)-isomer.

Blockade of the adrenergic α-receptors with phenoxybenzamine (bensylytum NFN) (1×10^{-6} g/ml for 30 min.) did not change the contractile action of mepivacaine 3×10^{-4} g/ml.

Electrophysiological effects of mepivacaine

In order to study to what extent the myogenic action of mepivacaine was correlated to the electrical events in the muscle membrane, experiments were performed with the sucrose-gap method. In normal Krebs solution the portal vein preparations showed a spontaneous rhythmic activity with bursts of action potentials accompanied by periods of increased tension as reported by FUNAKI & BOHR (1964). The waves of activity were separated by periods of relaxation during which both electrical and mechanical activities were absent (fig. 3a).

When DL-mepivacaine was added to a final concentration of 1×10^{-4} g/ml the tension during the bursts of activity increased. The resting membrane potential was not significantly changed (fig. 3b).

When the concentration of DL-mepivacaine was increased to 3×10^{-4} g/ml or more a continous electrical activity appeared (fig. 3c). The rapid spike discharge was accompanied by an increased tension. In some experiments the spike amplitude was slightly increased (fig. 3c, 5'). Mepivacaine still had no significant effect on the resting membrane potential. The small changes in the membrane potential were all within the limits of error of the sucrose-gap method. The tension and spike discharge

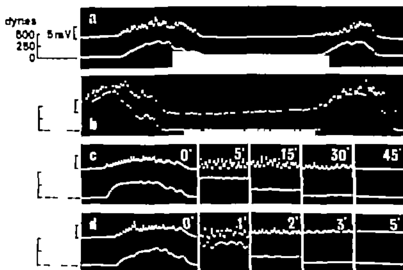


Fig. 3 Sucrose-gap registrations of the effects of DL-mepivacaine on electrical (upper tracings) and mechanical (lower tracings) activities in the rat portal vein. (a) Spontaneous activity in normal solution. (b) The same vessel as (a), but after 10 min. exposure to DL-mepivacaine 1×10^{-4} g/ml. (c) 0' shows the spontaneous activity 5' 15' 30' and 45' are registrations taken at these times (min.) from the addition of DL-mepivacaine 3×10^{-4} g/ml. (d) Same as (c) but DL-mepivacaine was used in the concentration 3×10^{-6} g/ml.

reached its maximum 5–10 min. after the addition of DL-mepivacaine 3×10^{-4} g/ml (fig. 3c, 5'). Thereafter the tension decreased (fig. 3c, 15') and finally disappeared in spite of a persisting high spike activity (fig. 3c, 30'). The spike activity decreased after about 15 min. and disappeared after about 45 min. (fig. 3c, 45').

In a concentration of 3×10^{-6} g/ml the relaxing action of mepivacaine was initially accompanied by frequent action potentials. Thus there was initially a dissociation between the electrical and mechanical activities. After a few minutes, however the frequency of action potentials was decreased and in many experiments there were no action potentials after 5–15 min. (fig. 3d).

On repeated additions of DL-mepivacaine 3×10^{-4} g/ml every 30 min there were no further electrical or mechanical effects of the drug on the isolated blood vessels after the first dose.

When the portal veins had been washed with fresh Krebs solution after experiments with DL-mepivacaine 3×10^{-4} g/ml, the normal phasic activity was restored within 15 min.

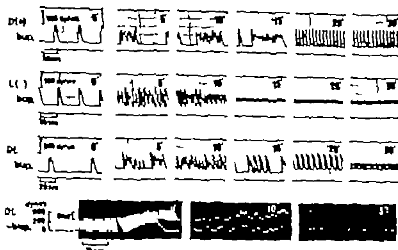


Fig. 4 Effects on the rat portal vein of the isomers and the racemate of bupivacaine in the concentration 5×10^{-6} g/ml.
The three uppermost stripes show only the mechanical activity while the lowest strip is a sucrose-gap registration of both mechanical and electrical activities. The times (min.) of exposure to the different test compounds are marked in the figure.

Mechanical and electrophysiological effects of bupivacaine.

In a concentration of 1×10^{-6} g/ml there were no mechanical or electrical effects of DL- L(−) or D(+)-bupivacaine on the rat portal vein in normal Krebs solution.

In contrast to mepivacaine the maximal tension produced by bupivacaine in the dose range 1×10^{-5}–1×10^{-4} g/ml did not exceed that of the spontaneously produced tension (fig. 6). The myogenic activity of the vein was however changed by bupivacaine. In a concentration of 5×10^{-6} g/ml the frequency of contractions was markedly increased both by the



Fig. 5. Responses of an isolated rat portal vein to DL-bupivacaine. (a) Spontaneous activity in normal solution. (b) After 5 min. exposure to DL-bupivacaine 1×10^{-4} g/ml. (c) After 15 min. exposure to DL-bupivacaine 1×10^{-4} g/ml. (d) After 60 min. exposure to DL-bupivacaine 1×10^{-4} g/ml.

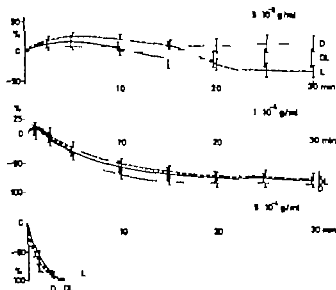


Fig. 6. Effect of DL- L(−)- and D(+)-bupivacaine on the tension in the rat portal vein. Changes of the tension are expressed as per cent of the maximal tension of the individual muscles during spontaneous activity. Each curve shows mean ± S.E.M. from 8 muscles.

isomers and the racemate (fig. 4). High frequency contractions appeared within 30 min. in 7 out of 9 experiments with DL-bupivacaine, in all 9 experiments with L(−)-bupivacaine and in 5 out of 9 experiments with D(+)-bupivacaine 5×10^{-3} g/ml. In the experiments in which high-frequency contractions appeared, the frequency per min. was 31 ± 6, 34 ± 4 and 15.5 ± 3 after 30 min. exposure to DL L(−) and D(+)-bupivacaine. The difference between the isomers was significant ($P < 0.05$), while the racemate did not differ significantly from any of the isomers ($P > 0.05$). When racemic bupivacaine or the isomers of bupivacaine were used in the concentration 1×10^{-4} g/ml, an increased frequency of action potentials was seen in sucrose-gap experiments (fig. 5). The high-frequent action potentials were sometimes accompanied by a short initial phase of increased contractility. After a few minutes there was always a decreased contractility by the bupivacaine compounds in this concentration (fig. 5c, d). A dissociation between electrical and mechanical activities in the portal vein was seen during relaxation (fig. 5d). The frequency of action potentials decreased after 30–60 min. and after 90–150 min. the action potentials had disappeared. There was however no significant difference between the effects of the isomers of bupivacaine in the concentration 1×10^{-4} g/ml.

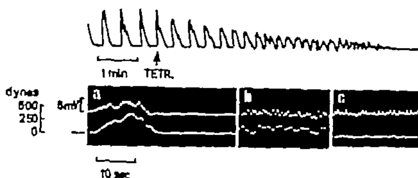


Fig. 7 Effects of tetracaine on mechanical and electrical activities in the rat portal vein. The upper part of the figure is a registration of the immediate mechanical effects of tetracaine 1×10^{-4} g/ml. The lower part of the figure shows registrations from a sucrose-gap experiment. (a) Spontaneous activity in normal solution, (b) After 5 min. exposure to tetracaine 1×10^{-4} g/ml (c) After 8 min. exposure to tetracaine 1×10^{-4} g/ml.

In the concentration 5×10^{-4} g/ml the racemate and the isomers of bupivacaine relaxed the muscle very quickly and without any preceding contractile phase. In this concentration the mechanical and electrical effects of the bupivacaine compounds were very much like those of tetracaine in the concentration 1×10^{-4} g/ml (fig. 7).

The racemate and the isomers of bupivacaine did not influence the resting membrane potential in the concentration range 1×10^{-6} to 5×10^{-4} g/ml.

In experiments with DL-bupivacaine 1×10^{-4} g/ml the normal phasic activity of the portal veins was not restored until 25–35 min. after the drug had been washed out.

Mechanical and electrophysiological effects of tetracaine.

Tetracaine in a concentration of 1×10^{-4} g/ml decreased the tension but increased the frequency of the contractions in the portal vein (fig. 7). After a few minutes the electrical and mechanical activities were almost continuous (fig. 7b). After another two or three minutes (fig. 7c) there were single action potentials with a low amplitude (about 2 mV) The single-spike activity was not always accompanied by any mechanical activity The tissue was completely relaxed within 10 min. after tetracaine had been added to the bath fluid to a final concentration of 1×10^{-4} g/ml (fig. 7).

Tetracaine (1×10^{-4} g/ml) completely blocked the electrical and mechanical responses of DL-mepivacaine (3×10^{-4} g/ml) in the isolated portal veins when tetracaine was added to the bath fluid 15 min. before mepivacaine.

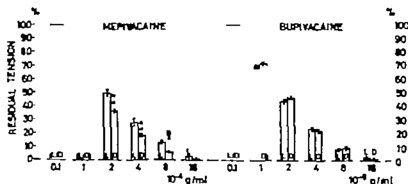


Fig. 8. Residual tension in the noradrenaline-contracted (1×10^{-6} g/ml of noradrenaline) rat portal vein after addition of increasing doses of the isomers of mepivacaine and bupivacaine. The bars show mean ± S.E.M. from 8 experiments.
The significance of the difference between the isomers is shown in the figure, where means $P < 0.01$.

Effects of local anaesthetics on contracted portal veins

The relaxing action of the isomers of mepivacaine and bupivacaine were compared on portal veins contracted with noradrenaline in a concentration of 1×10^{-6} g/ml. Both isomers of mepivacaine had a dose-dependent relaxing action in the range 1×10^{-6}–1.6×10^{-4} g/ml. The D(−)-isomer was significantly more active than the L(+)-isomer (fig. 8). The isomers of bupivacaine were about 10 times more potent than those of mepivacaine. There was however no significant difference between the activity of the isomers of bupivacaine (fig. 8).

To test the relaxing action of the isomers of mepivacaine on another type of contraction (VAN BREEMEN & LESSER 1971), the muscle membrane was



Fig. 9. Sucrose-gap registration of the relaxing effect of DL-mepivacaine on K depolarized rat portal vein. (a) Immediate effect of K-high Krebs solution. (b) After 5 min. exposure to the K-high solution. (c) Activity 5 min. later and 4 min. after adding DL-mepivacaine to a final concentration of $\times 10^{-4}$ g/ml in the K-high Krebs solution.

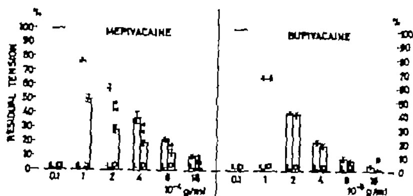


Fig. 10. Residual tension in the K-contracted (128 mM K^+) rat portal vein after addition of increasing doses of the isomers of mepivacaine and bupivacaine. The bars show mean ± S.E.M. from 8 experiments.
The significance of the difference between the isomers is shown in the figure, where *** means $P < 0.001$, means $P < 0.01$ and means $P < 0.05$.

depolarized in a "K-high" Krebs solution. The high concentration of K-ions (128 mM) caused an immediate depolarization, a complete cessation of the spike discharge and an increase in tension (fig. 9). There was no contracting effect of the local anaesthetics tested on the depolarized portal veins. The relaxing action of the isomers of mepivacaine was as marked on the K-contracted muscles as on muscles contracted with noradrenaline, the D(−)-isomer being much more active than the L(+)-isomer (fig. 10).

Discussion

The myogenic action of the local anaesthetic agents studied on the rat portal vein was both dose- and time-dependent and there were clear differences in activity between the agents and also between their isomers.

The effects of the compounds investigated on the isolated smooth muscle consisted of two components.

a) In the lowest active concentrations the tension and/or the frequency of contractions were increased.
b) In higher concentrations there was a relaxing action of the local anaesthetics.

The ultimate effect on the mechanical activity reflected the sum of the contracting and relaxing abilities of the compounds.

L(+)-mepivacaine, but not D(−)-mepivacaine had a contracting action on the normal portal vein in the concentration 1×10^{-4} g/ml (fig. 2) in

which concentration none of the isomers had any relaxing action (fig. 8 & 10). We therefore suggest that L(+)-mepivacaine is more potent in stimulating the contractile components than the D(−)-isomer.

The contracting action of the compounds tested was undoubtedly trigged by the spike activity. It is therefore probable that the contracting properties of the compounds were eliminated in the depolarized muscle, thus making possible a direct comparison of their potencies to stimulate the relaxing components of the muscle: as shown in fig. 8 & fig. 10 the D(−)-isomer of mepivacaine was significantly more potent in relaxing the muscle than the L(+)-isomer.

The differences in the myogenic activity between the two isomers of mepivacaine, shown in fig. 2, were therefore dependent on a twofold action, namely

1. the L(+)-isomer was most potent in stimulating the contractile components of the muscle.
2. the D(−)-isomer was most potent in stimulating the relaxing components.

The myogenic activity of the racemate was mainly like that of the L(+)-isomer indicating that the contracting activity of the L(+)-isomer was comparatively more dominant than the relaxing activity of the D(−)-isomer.

That bupivacaine and its optically active isomers did not increase the tension of isolated portal veins (fig. 6) despite an increased frequency of contractions, may be explained on the basis of the marked relaxing effects of the compounds (fig. 8, 10). A corresponding relationship between the contracting and relaxing activities, the latter dominating over the former, probably was present for tetracaine too. We therefore suggest that the strong vasoconstrictor action of L(+)-mepivacaine, in relation to that of the other local anaesthetic agents, is a function both of its potent contractile action and of its rather weak relaxing action on the smooth muscle.

The actions of mepivacaine on the isolated rat portal vein were very similar to those found *in vivo*. Thus DL-mepivacaine had a contracting action on the femoral vascular bed of the anaesthetized dog when the vascular tone was low, and a relaxing action on the same vascular bed when the vascular tone was high (ÅBERG & DHUNÉR 1972). When the vascular effects of infiltrations with mepivacaine and its isomers were studied in man by means of a thermographic method it was found that L(+)-mepivacaine decreased the skin temperature more than D(−)-mepivacaine (ÅBERG & ADLER 1970). This indicates a stronger vasoconstrictor effect of L(+)-mepivacaine than of D(−)-mepivacaine *in vivo*, which is in accordance with the present results on the rat portal vein *in vitro*.

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From the Department of Pharmacology AB Bofors, Nobel-Pharma,
S-431 33 Mölndal, Sweden, and the First Department of Anaesthesia,
Sahlgrenska Sjukhuset, Göteborg, Sweden

Effects of Mepivacaine (Carbocaine®) on Femoral Blood Flow in the Dog

By

G. Åberg and K.-G. Dhunér

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Abstract: The vascular effects of the local anaesthetic compound mepivacaine were investigated on the femoral blood flow in anaesthetized dogs. The vascular resistance was increased by intraarterial infusions of mepivacaine when the initial vascular tone was low. When the initial vascular resistance was high, mepivacaine had a vasodilating effect. This was not caused by blockade of the sympathetic nerves, but was attributed to direct action on the vascular smooth muscle. The vasoconstricting and vasodilating actions of mepivacaine were in accordance with results from tests on isolated vascular smooth muscle.

Key-words: Local anaesthetics – vascular effects.

Using the ^{133}Xe clearance technique DHUNÉR & LEWIS (1966) have demonstrated a local vasodilating effect of mepivacaine (carbocaine® Bofors Nobel Pharma) when injected intramuscularly in man. Several other investigators using different techniques have reported vasoconstrictor effects by the same local anaesthetic agent (PORTO & SCHEININ 1958, DU MESNIL DE ROCHEMONT & HENSEL 1960, ADLER *et al.* 1969, ÅBERG & ADLER 1970, JORFELDT *et al.* 1970). Recently ÅBERG & WAHLSTRÖM (1972) have shown *in vitro* that mepivacaine in a wide range of concentrations (1×10^{-8}–1×10^{-4} g/ml) had a contracting effect on relaxed vascular smooth muscle, while the compound in the same concentrations had a relaxing effect on contracted vascular smooth muscle. In a high concentration ($> 1 \times 10^{-4}$ g/ml) mepivacaine was found to relax spontaneously active smooth muscle.

Vasodilatation elicited by procaine has been observed by SANDERS (1965) in the hind limb of spinal cats when the basal tone was high, when the basal tone was low procaine produced vasoconstriction.

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Table 1

Vascular effects of intraarterial infusions of mepivacaine into the femoral artery of dogs.
Mepivacaine was infused during 2 min. periods.
All values are means ± S.E.M.
The significance of the differences from the control values are shown in the table, where means $P < 0.05$ and means $P < 0.01$.

Pretreatment	Dose of mepivacaine mg/kg/min.	Number of dogs	Number of tests	Control			During infusion of mepivacaine		
				Blood pressure mmHg	Femoral blood flow ml/min.	Femoral vasc. resist.	Blood pressure mmHg	Femoral blood flow ml/min.	Femoral vasc. resist.
None	1.0	5	7	133 ± 4.3	89 ± 6.7	1.5 ± 0.1	133 ± 4.3	113 ± 7.0*	1.2 ± 0.1
None	3.0	6	9	128 ± 4.4	83 ± 5.4	1.6 ± 0.1	126 ± 4.5	136 ± 10.5**	1.0 ± 0.1
None*)	1.0	3	6	106 ± 3.8	139 ± 4.4	0.8 ± 0.02	106 ± 3.8	92 ± 9.1**	1.2 ± 0.1
Noradrenaline 0.1 μg/kg/min.	1.0	6	13	140 ± 7.2	91 ± 5.5	1.6 ± 0.1	127 ± 5.6	129 ± 8.9**	1.0 ± 0.1
Dibenzyline 5–7.5 mg/kg	1.0	6	11	76 ± 3.6	58 ± 4.8	1.3 ± 0.1	78 ± 3.3	43 ± 4.6	1.9 ± 0.1**

*) Femoral sympathetic innervation damaged during preparation (?).

fusion of mepivacaine 1 mg/kg/min. caused a significant decrease in the femoral vascular resistance, resulting in a significantly increased femoral blood flow (table 1)

Effect of mepivacaine after adrenergic α-receptor blockade

When the sympathetic tone had been decreased with dibenzyline 5–7.5 mg/kg, mepivacaine caused a significant increase of the femoral vascular resistance and a significantly decreased femoral blood flow (table 1)

Discussion

Mepivacaine, and particularly its L(+)-isomer has been shown to have a marked vasoconstrictor effect on the isolated rat portal vein (ÅBERG & WAHLSTRÖM 1972) According to the terminology of BOZLER (1948) this tissue has a single-unit organization of the smooth muscles (FUNAKI & BOHR 1964 JOHANSSON & BOHR 1966) like that of peripheral resistance vessels (JOHANSSON & BOHR 1966) In the experiments *in vitro* the contracting effect of mepivacaine was only seen when the isolated vessel had a low initial tone and when mepivacaine was used in a concentration range from 1×10^{-6} g/ml to 1×10^{-4} g/ml (ÅBERG & WAHLSTRÖM 1972). In the experiments reported in this paper there was a vasoconstrictor effect *in vivo* by mepivacaine within the same range of concentration as that shown to contract vascular smooth muscle *in vitro* In accordance with the *in vitro* experiments the contraction produced by mepivacaine *in vivo* only occurred when the initial vascular resistance was low (table 1) As the vasoconstriction by mepivacaine *in vivo* was not inhibited by adrenergic α-blockade (table 1) it was probably not caused by a blockade of the uptake mechanism for noradrenaline, which has been described for cocaine (MUSCHOLL 1961).

JORFELDT *et al.* (1970) have reported an increased vascular tone in capacitance vessels of human forearms during intraarterial infusions of mepivacaine. In the *in vitro* experiments of ÅBERG & WAHLSTRÖM (1972) the isolated rat portal vein was contracted by mepivacaine in the same concentrations as those used by JORFELDT *et al.* (1970). JORFELDT *et al* (1970) also found an increased tone in the resistance vessels after intraarterial infusions of very small doses of mepivacaine. This effect, however was eliminated by a stellate ganglion block and was suggested by the authors to be due to a "cocaine-like" action of mepivacaine i.e. blockade of noradrenaline-uptake by the sympathetic nerve endings.

The vasodilating effect of mepivacaine, which was recorded during some of our experiments (table 1) is in good agreement with the results of other authors, e.g. NISHIMURA *et al.* (1965) who also injected mepivacaine intra-

arterially into the peripheral circulation of animals. In their experiments and in those reported by DHUNÉR & LEWIS (1966), the vascular tone was decreased by mepivacaine either by a direct relaxing effect on the vascular smooth muscles or by a local sympathetic nerve blocking action of the compound. The vasodilating effect of mepivacaine reported in this paper was also present in those experiments where a high vascular tone was maintained by a continous intravenous infusion of noradrenaline. The vasodilating effect of mepivacaine in our experiments was therefore not the result of a sympathetic nerve block, but had presumably the same mechanism as the one reported from *in vitro* experiments on the contracted rat portal vein (ÅBERG & WAHLSTRÖM 1972, ÅBERG & ANDERSSON 1972).

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From the Department of Pharmacology AB Bofors Nobel-Pharma,
431 33 Mölndal, Sweden

Toxicological and Local Anaesthetic Effects of Optically Active Isomers of Two Local Anaesthetic Compounds

By

G. Åberg

(Received November 4, 1971 Accepted November 26, 1971)

Abstract. Mepivacaine. There was no difference in toxicity between the two optical isomers of mepivacaine after rapid intravenous injections into mice or rats. After slow intravenous injections or after subcutaneous injections the L(+)-isomer however was less toxic than the D(−)-isomer This might at least partly be explained by the results obtained from other experiments, which demonstrated that after slow infusions of the drugs into rabbits more L(+)-mepivacaine than D(−)-mepivacaine was taken up by the lungs, thus reducing the concentration of the drug reaching the brain, where significantly more D(−)-mepivacaine than L(+)-mepivacaine was found. A slow absorption of L(+)-mepivacaine in comparison with D(−)-mepivacaine from the site of injection is demonstrated and might contribute to the differences in subcutaneous toxicity and might also be the cause of the significantly longer duration of infiltration anaesthesia by the L(+)-isomer than by the D(−)-isomer since the same nerve-blocking effect of the isomers was found *in vitro*. *Bupivacaine:* There were significant differences between the toxicity of the bupivacaine isomers when given intravenously and subcutaneously the D(+)-isomer was more toxic. This as well as the very long duration of infiltration anaesthesia by L(−)-bupivacaine indicate differences in absorption rates between the bupivacaine isomers similar to those demonstrated for the mepivacaine isomers.

Key words: Local anaesthetics – optical isomers – toxicity efficiency – absorption distribution.

Several investigators have reported differences in the nerve-blocking ability between optical isomers of local anaesthetic compounds (GOTTLIEB 1923 SCHÖNENBERGER *et al.* 1967 AKERMAN *et al.* 1967 & 1969) This report concerns same basic toxicological and local anaesthetic effects of the optical isomers of mepivacaine (carbocaine® Bofors) and bupivacaine (marcaine® Bofors) in animals. Previous reports on these isomers have been given by LUDUENA (1969) and from our laboratories by AF EKENSTAM *et al.* (1957),

ÅBERG & WAHLSTRÖM (1969) ÅBERG & ADLER (1970) ADLER *et al.* (1969) and by FRIBERGER & ÅBERG (1971). The dextro- and levoforms of the presently investigated local anaesthetics are found to have the same nerve-blocking ability *in vitro* but there were significant differences between some of their toxicological and local anaesthetic effects *in vivo*. These isomers may thus prove to be valuable tools for an analysis of factors that influence the biological effects of local anaesthetics *in vivo*.

Methods

Acute toxicity

a. *Intravenous toxicity in mice and rats.*

The intravenous toxicity was determined on female NMRI mice weighing 23 ± 2 g and on female Sprague-Dawley rats weighing 70 ± 5 g. The local anaesthetics were dissolved in a NaCl-solution in concentrations so that 10 ml of the isotonic solutions per kg body weight was administered into the tail vein. The rates of injection were 0.01 ml/sec. and 0.1 ml/sec. respectively. The injections were performed by hand by a trained technician. LD50-values were calculated on the mortality rate observed 48 hours after the administration of the drugs by the method of MILLER & TAINTER (1944).

b. *Intravenous toxicity in rabbits.*

Isotonic solutions of the test compounds were administered by a constant speed infusion pump into a marginal vein of the ear of male albino rabbits weighing 2000 ± 200 g. The infusion speed was 0.32 ml/min. The infusions were continued until the animals died (cardiac arrest). The mean convulsive dose (CD mean) and the mean lethal dose (LD mean) were calculated.

c. *Subcutaneous toxicity in mice and rats.*

Female NMRI mice weighing 23±2 g and female Sprague-Dawley rats weighing 70 ± 5 g were used. The test solutions were made isotonic by the addition of NaCl. The injected volumes were 10 ml/kg into the mice and 20 ml/kg into the rats and the solutions were injected subcutaneously into the caudal part of the back. The LD50-values were determined as described above.

Local anaesthetic action.

a. *Nerve blocking effects in vitro*

Following the procedure of ÅBERG & WELIN (1967) the nerve blocking activity of the test compounds was studied on isolated sciatic nerve preparations of frogs (*Rana temporaria*). In short, the nerves were pulled through a T-shaped glass tube which was perfused with a frog Ringer solution containing the test solutions. The nerves were stimulated with 0.8 V and 0.2 msec. and the propagated action potentials ("spikes") were recorded on an oscilloscope. The spike amplitude, in mV was noted every minute until it was stabilized and then every fifth minute during the recovery period, during which the nerves were continuously washed with fresh frog Ringer solution. As the second and third block of the isolated nerve develop much faster than the first one, the nerve was not used for more than one test (BROWN & LUDUENA 1953).

After application of a test solution, the amplitude of the action potentials decreased

to a stable value. The spike amplitude was then calculated as a per cent of the initial spike amplitude. The mean ± S.E.M. of five such determinations was taken as the nerve blocking effect of a test solution. The experiments were performed in a thermostable room at 20

b. *Nerve blocking effects in vivo.*
The hair of the posterior part of the back of female Sprague-Dawley rats weighing 160 ± 10 g was clipped off. The animals were slightly restrained and a No. 16 hypodermic needle was introduced between the dorsal spine and the trochanter to a depth of about 2 mm, and 0.25 ml of an isotonic test solution was injected. Both motor and sensory blocks were observed. The paralysis of the hindleg was taken as a sign of motor nerve block and was tested when the animal was forced to walk. Sensory blocks were studied using an artery clip that was applied to the paw. The nerve blocks were tested every minute until complete anaesthesia was achieved and then every fifth minute. The time between the injection and the onset of anaesthesia was called "time of onset" and was calculated as the time from the injection until the animal showed complete sensory resp. and motor nerve block. The duration of anaesthesia was calculated as the time from the onset of anaesthesia until the animal showed no sign of hind-leg paralysis and showed a normal pain reaction to the application of the artery clip. The experiments were performed in a "blind" manner using randomized test solutions.

c. *Infiltration anaesthesia.*
In female guinea-pigs weighing 400±50 g, the back was depilated on the day before the experiment. The naked surface was divided into four equally large fields with Indian ink. In the centre of each field 0.20 ml of the test solution from one of the randomized ampoules was injected. The anaesthesia was checked by needle algesimeter six pricks on the wheal area every minute until complete anaesthesia was achieved and then every tenth minute. The "time of onset" was calculated as the time from the injection until three out of six pricks were not felt by the animal. The duration of anaesthesia was calculated as the time from the onset of anaesthesia until three out of six pricks were distinctly felt during recovery from the local anaesthetic effect. The experiments were performed in a thermostable room (24°) where the animals had been caged for at least 24 hours.

This method is a slight modification of the method of BÜLBRING & WAJDA (1945) and HINGY (1960).

d. *Rate of absorption.*
Tritiated isomers and racemate of mepivacaine were injected intradermally into female guinea-pigs weighing 170±10 g. The concentration of the injected solutions was 0.5%. In one series of experiments the animals were killed by stunning and bled immediately before the start of the intradermal injections. In other experiments the injections were performed on live animals. The injected volume was 0.20 ml. Only one injection was given to each animal. At prefixed times after the injections the live animals were rapidly killed and a piece of the dorsal skin containing the injection site was rapidly cut out. A circular part (~250 mm²) of the skin around the site of injection was then excised by means of a cork-borer. Dorsal skin flaps containing the injection sites were obtained from the pre-killed animals in the same way. The excised skin flaps were then dissolved in 2 ml of soluene and after the addition of 10 ml of a PPO-POPOP solution containing 3 g diphenyl-oxazol (PPO) and 0.3 ml 2,2-p-phenylene-bis-

(5-phenyloxazole) (POPOP) in 1 l toluol, the radio-activity was measured in a Packard Tri-Carb Scintillation Counter

The local anaesthetic activities of the test solutions were investigated in parallel to the isotope experiments, according to the modified method BÜLBRING & WAJDA (1945) already described. The tests of local anaesthesia were performed on guinea-pigs of the same sex and weight as the isotope experiments and in most of these tests too only one injection was given to each animal. The experiments were performed at room temperature (22°). The pH of the isotonic and sterile test solutions was 5.4–5.5 This method of absorption study is a modification of the original method of SECHER-HANSEN et al. (1967).

e. Distribution in rabbits

The test solutions were administrated intravenously to male unanaesthetized rabbits, weighing 1.4–1.6 kg. Each of the isomers and the racemate of mepivacaine were administrated to each of 6 animals. The total dose was 5.0 mg/kg body weight and this was given in an isotonic, unbuffered solution (1.0 ml/kg body weight) during 60 sec. by means of an infusion pump into a marginal ear vein. The animals were rapidly killed by stunning 2 min. after the cessation of the infusion and the arterial blood was immediately collected from the thoracic aorta which was exposed by a quickly performed thoracotomy The cerebral hemispheres, the cerebellum, the lungs, the kidneys, the liver the heart and the salivary glands were also excised, weighed and analyzed for mepivacaine content.

The quantitative estimations of the tissue concentrations of mepivacaine were carried out by a gaschromatographic method, developed in our analytical department (HARTHON & WIKSTRÖM, personal communication).

Results

Acute toxicity

Intravenous toxicity in mice and rats

When given intravenously at an injection speed of 0 01 mg/sec. to mice, the LD50-values for the D-isomers of mepivacaine and bupivacaine were lower than the corresponding LD50-values for their enantiomers (*table 1*). There were no differences between the LD50-values of the isomers of mepivacaine after intravenous injections into mice and rats at an injection speed of 0 1 ml/sec. but after slow injections (0 01 ml/sec.) to mice, L(+)−mepivacaine was less toxic than D(−)−mepivacaine (table 1). The LD50-value of the D(+)−isomer of bupivacaine was lower than the corresponding value of the L(−)−form when given intravenously at 0 1 ml/sec. to both mice and rats and also when given at 0 01 ml/sec. to mice (table 1).

Intravenous toxicity in rabbits

There were significant differences in the mean lethal doses of the mepivacaine isomers ($P < 0.05$) and between the bupivacaine isomers ($P < 0.01$) during slow intravenous infusions into rabbits (table 2), the D-isomers being

Table 1

Intravenous toxicity in mg/kg body weight of the racemic forms of mepivacaine and bupivacaine and their optical isomers. The injected volume in both species was 10 ml/kg body weight.

Compound	LD50 (± ε) Mice 0.1 ml/sec.	Mice 0.01 ml/sec.	Rats 0.1 ml/sec.
DL-mepivacaine	35 ± 3.0 (n = 36)	44 ± 2.0 (n = 20)	35 ± 4.0 (n = 36)
L(+)−mepivacaine	34 ± 3.5 (n = 36)	49 ± 2.0 (n = 30)	37 ± 3.0 (n = 36)
D(−)−mepivacaine	32 ± 2.5 (n = 36)	40 ± 3.0 (n = 50)	36 ± 3.5 (n = 36)
DL-bupivacaine	7.3 ± 1.0 (= 36)	10 4 ± 0.5 (n = 20)	5.6 ± 0.2 (n = 36)
L(−)−bupivacaine	9.6 ± 1.0 (n = 36)	11.1 ± 0.6 (n = 40)	7.2 ± 0.4 (= 36)
D(+)−bupivacaine	7.9 ± 1.0 (n = 36)	7.6 ± 0.4 (n = 40)	3.8 ± 0.2 (n = 36)

the more toxic (table 2). D(+)−bupivacaine caused convulsions in the rabbits in significantly ($P < 0.01$) lower doses than L(−)−bupivacaine, but there were no significant differences ($P > 0.1$) between the convulsive doses of the mepivacaine isomers (table 2).

c. Subcutaneous toxicity in mice and rats

There were great differences in LD50-values between the mepivacaine isomers and between the bupivacine isomers after subcutaneous injections in mice the D-isomers were more toxic (table 3) There was however no difference in subcutaneous toxicity in rats between the mepivacaine isomers.

Table 2

Convulsive and lethal doses of local anaesthetics following intravenous administration into rabbits.

Compound	Number of tested animals	Infusion speed mg/kg/min.	Convulsive dose (mg/kg) Mean ± S.E.M.	Lethal dose (mg/kg) Mean ± S.E.M.
DL-mepivacaine	8	5	22 ± 2.0	53 ± 3 0
L(+)−mepivacaine	8	5	20 ± 2.0	48 ± [illegible].0
D(−)−mepivacaine	9	5	18 ± 1.0	38 ± 3.0
DL−bupivacaine	8	1	3.3 ± 0.3	6.9 ± 0.7
L(−)−bupivacaine	8	1	4.7 ± 0.4	9 7 ± 0.8
D(+)−bupivacaine	8	1	2.7 ± 0.3	5.5 ± 0.3
Lidocaine	8	5	14 ± 1.0	41 ± 2.0
Tetracaine	8	1	2.6 ± 0.2	6.0 ± 0.2

Table 3

Subcutaneous toxicity as mg/kg body weight of racemates and isomers of mepivacaine and bupivacaine.

Compounds	LD50 (± ε) Mice	Rats
DL−mepivacaine	280 ± 33 (n = 30)	500 ± 53 (n = 48)
L(+)−mepivacaine	330 ± 44 (n = 30)	530 ± 48 (n = 48)
D(−)−mepivacaine	175 ± 21 (n = 30)	500 ± 50 (n = 60)
DL−bupivacaine	53 ± 5 (n = 36)	48 ± 3 (n = 40)
L(−)−bupivacaine	100 ± 9 (n = 36)	52 ± 4 (n = 40)
D(+)−bupivacaine	30 ± 3 (n = 36)	33 ± 3 (n = 40)

Nerve blocking effect in vitro

There were no significant differences between the nerve blocking potency *in vitro* of the L– DL– and D–forms of mepivacaine or bupivacaine (fig 1).

Nerve blocking effects in vivo

The durations of the sciatic nerve blocking effects of the compounds investigated are given in fig. 2 and 3

Among the mepivacaine compounds (fig. 2) there were no statistically significant differences between the anaesthetic effects when given in low con-

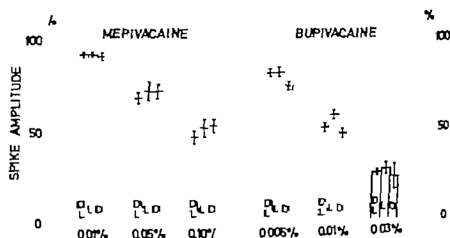


Fig. 1. Nerve blocking effects (mean ± S.E.M.) of the DL–, L– and D–forms of mepivacaine and bupivacaine *in vitro*. Each bar shows the mean ± S.E.M. from 5 determinations.

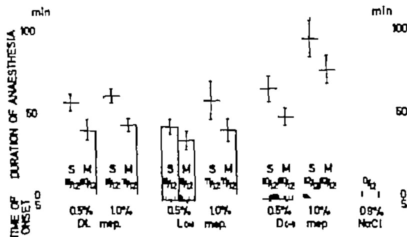


Fig. 2. Sciatic nerve blocks in rats after injections of DL−, L(+)− and D(−)−mepivacaine. S = sensory nerve block; M = motor nerve block. The frequency of successful anaesthesias is given within the bars. N = 12.

centrations (0.5 /). In a higher concentration (1.0%) the D(−)−form gave a longer nerve blocking effect than both the L(+)−form and the racemate ($P < 0.01$ for motor blocks and $P < 0.001$ for sensory blocks).

In these tests there were no statistically significant differences between the observed effects of the bupivacaine compounds (fig. 3).

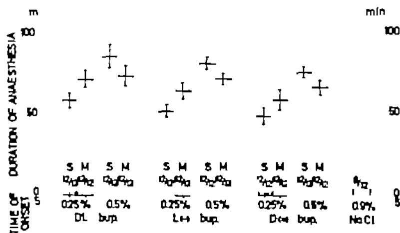


Fig. 3. Sciatic nerve blocks in rats after injections of DL−, L(−)− and D(+)−bupivacaine. S = sensory nerve block; M = motor nerve block. The frequency of successful anaesthesias is given within the bars. N = 12.

Infiltration anaesthesia.

Solutions without vasoconstrictors (fig. 4).

For all compounds and for all the tested concentrations, the time of onset of the anaesthesia, effect was less than 1 min. In the lowest concentration (0.25 %) there were no significant differences between the effects of the isomers of mepivacaine. When the concentrations were higher (0.5 % and 1.0 %) the L(+)−form of mepivacaine gave significantly ($P < 0.02$) longer durations of anaesthesia than the D(−)−form.

In all the investigated concentrations (0.05 %, 0.10 % and 0.20 %) L(−)−bupivacaine gave a significantly more prolonged duration of anaesthesia than D(+)−bupivacaine ($P < 0.001$). In the highest concentration (0.25 %) the anaesthetic effect of the racemate differed significantly both from that of L(−)−bupivacaine ($P < 0.01$) and that of D(+)−bupivacaine ($P < 0.001$)

Solutions with vasoconstrictors (fig. 5).

When adrenaline was added to the test solutions in a concentration of 5×10^{-6} g/ml there was still a significant difference between the durations of the local anaesthetic effects of the mepivacaine isomers ($P < 0.01$). In this test there was however no significant difference ($P > 0.05$) between the effects of the bupivacaine isomers in the presence of adrenaline.

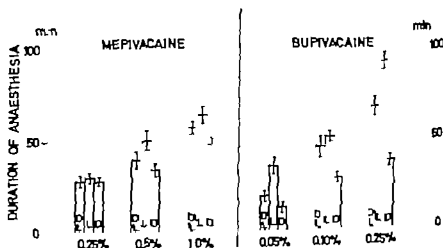


Fig. 4. Duration of anaesthesia (mean ± S.E.M.) of mepivacaine, bupivacaine and the optically active isomers of the compounds in intradermal wheal tests (infiltration anaesthesia) in guinea pigs. The frequency of anaesthesia was 0/12 in the placebo group (0.9 % NaCl) in the mepivacaine test as well as in the bupivacaine test. The pH of the test solutions was 5.5–5.8. N = 12.

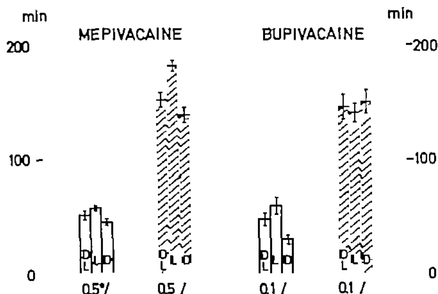


Fig. 5. Effect of adrenaline 5 μg/ml on the duration of anaesthesia (mean ± S.E.M.) of mepivacaine, bupivacaine and the optically active isomers of the compounds in intradermal wheal tests (infiltration anaesthesia) in guinea pigs. Open bars = plain solutions: striped bars = solutions with adrenaline 5 μg/ml. The frequency of anaesthesia was 0/12 in the placebo group (0.9% NaCl) in both the mepivacaine test and the bupivacaine test. The pH of the test solutions was 4.0–4.2. N = 12.

Rate of absorption.

The results of the experiments in which solutions of tritiated DL – L(+) – and D(–)–mepivacaine were injected intradermally in to guinea-pigs show that D(–)–mepivacaine was absorbed significantly faster than L(+)–mepivacaine (fig. 6) The durations of the intradermal infiltration anaesthesias of 0.5 / solutions were tested in parallel to the absorption tests on live animals and were 40 ± 5 min. for DL–mepivacaine, 50 ± 6 for L(+)–mepivacaine and 35 ± 3 min. for D(–)–mepivacaine.

Distribution in rabbits

The distribution of the isomers and the racemate of mepivacaine two minutes after an intravenous infusion of 5 mg/kg body weight is shown in fig. 7 In the lungs, there was significantly ($P < 0.001$) more L(+)–mepivacaine than D(–)–mepivacaine The same relationship was found in the kidneys, where there was also significantly ($P < 0.05$) more L(+)–mepivacaine than D(–)–mepivacaine

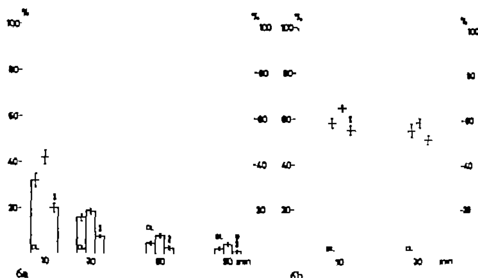


Fig. 6. Residual radioactivity in excised intradermal wheals, expressed as per cent of the injected radioactivity at different times after intracutaneous injections of 0.2 ml of tritiated solutions of DL–, L(+)– and D(–)–mepivacaine to guinea-pigs. Fig. 6a shows results from experiments on live animals and fig. 6b results from the experiments on dead animals. The concentration of the test solutions was 0.50%. Each bar shows the mean ± S.E.M. from 6 wheals. Room temperature was 22° and pH of the solutions was 5.5. The significance of the differences between the isomers in this experiment are shown in the figure, where * means $P < 0.05$ and ** means $P < 0.01$.

There was however a significantly ($P < 0.05$) higher concentration of D(–)–mepivacaine than of L(+)–mepivacaine in the cerebellum.

Discussion

The observed differences between the action of the isomers of mepivacaine and between the isomers of bupivacaine are summarized in table 4.

Mepivacaine. During slow intravenous administration into mice and rabbits the L(+)–isomer was less toxic than the D(–)–isomer. The subcutaneous toxicity of the L(+)–isomer was also less than that of the D(–)–isomer. The reason for the difference in toxicity between the isomers can probably be explained by differences in the distribution and in the tissue affinity of the two isomers. The cause of death after the injection of mepivacaine — like other local anaesthetics — is respiratory paralysis which is assumed to be of central origin (BRATTSAND 1967). After the intravenous in-

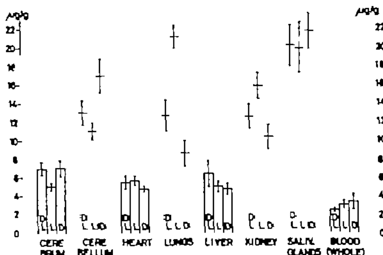


Fig. 7 Distribution of the racemate and the isomers of mepivacaine in rabbits, two minutes after the cessation of intravenous infusions of 5 mg/kg body weight of the drugs. Each bar shows the mean ± S.E.M. from 5 or 6 rabbits. The significance of the differences between the isomers is shown in the figure, where * means $P < 0.05$ and ** means $P < 0.001$.

fusion of L(+)−mepivacaine into young rabbits the concentration of mepivacaine in the cerebellum was significantly lower than in the animals given D(−)−mepivacaine the concentration in the lung was however higher (fig. 7).

The absorption of L(+)−mepivacaine after injections into the skin of guinea-pigs was slower than that of D(−)−mepivacaine (fig. 6) These facts might indicate that if the isomers are administered by slow intravenous infusion or if they gain access to the venous blood stream by subcutaneous injection, more of the L(+)− than of the D(−)−isomer is fixed to the lung tissue, thereby reducing the peak concentration reaching the brain tissue. On rapid intravenous injection there is less difference in toxicity between the isomers, which might indicate that in these experiments there is too little time for the L(+)−isomer to be fixed to the lung tissue to an extent that decreases the peak blood concentration sufficiently to reduce the brain concentration.

The duration of infiltration anaesthesia is more prolonged after the L(+)− than the D(−)−isomer both in the absence and presence of a vasoconstrictor This might be explained as the result of a higher tissue affinity of the L(+)−isomer than of the D(−)−isomer Moreover we found the L(+)−isomer of mepivacaine to have a much more pronounced vasocon

Table 4

Some differences in biological effects between the L- and the D-forms of mepivacaine and of bupivacaine.

Experimental conditions	Mepivacaine		Bupivacaine	
	L(+)−isomer	D(−)−isomer	L(−)−isomer	D(+)−isomer
Intravenous toxicity: slow injection (mouse, rabbit)	less toxic	more toxic	less toxic	more toxic
Subcutaneous toxicity (mouse)	less toxic	more toxic	less toxic	more toxic
Nerve blocks. efficiency *in vitro* (frog)	no difference		no difference	
Duration *in vivo* (rat)	shorter	longer	no difference	
Duration of infiltration anaesthesia (guinea-pig)				
Without vasoconstrictor	longer	shorter	longer	shorter
With vasoconstrictor	longer	shorter	no difference	
Absorption after intradermal injection (guinea-pig)	slower	faster	−*)	−*)
Tissue concentration after intravenous infusion (rabbit)				
Lungs	higher	lower	−*)	−*)
Cerebellum	lower	higher	−*)	−*)

*) not investigated.

strictor action than the D(−)−isomer both *in vivo* (ÅBERG & ADLER 1970) and *in vitro* (ÅBERG & WAHLSTRÖM 1972). In addition the D(−)−isomer has a stronger relaxing action than the L(+)−isomers both in vessels with spontaneous activity and in vessels contracted with adrenaline (ÅBERG & WAHLSTRÖM 1972). This difference in vasoactivity between the two isomers may contribute to or may be the main reason for the differences found between the effects of the two isomers in infiltration anaesthesia.

Satisfactory clinical effects in nerve blocks may be expected by local anaesthetics that have high penetration potencies and/or vasoconstrictor effects (SYDNES & ÅBERG, unpublished results). In the present experiments there

was a shorter time of onset, a higher frequency and a longer duration of anaesthesia by D(−)−mepivacaine than by L(+)−mepivacaine in rat sciatic nerve blocks (fig. 2) As vasoconstriction improves the time of onset, frequency and duration of anaesthesia (SYDNES & ÅBERG, unpublished results) the difference between the isomers in these tests cannot be attributed to the marked vasoconstrictor effect of L(+)−mepivacaine. The difference between the isomers might however be attributed to the difference between their rates of penetration which was demonstrated after intracutaneous injection into recently killed guinea-pigs, in which the effects of the isomers on the microcirculation were thus omitted (fig. 6b).

Bupivacaine. The differences between the effects of the isomers of bupivacaine were generally not as marked as those between the mepivacaine isomers. There were however significant differences between the bupivacaine isomers in the intravenous and subcutaneous toxicity tests the D(+)−isomer being most toxic (tables 1 2 and 3). There were almost no differences in the vascular effects between the isomers of bupivacaine (ÅBERG & WAHLSTRÖM 1972). The differences in toxicity as well as the very long duration of infiltration anaesthesia (fig. 4) after L(−)−bupivacaine therefore indicate differences in tissue penetrability between the isomers of bupivacaine like those demonstrated for the mepivacaine isomers.

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From the Institute of Pharmacology University of Oslo, Blindern, Oslo 3 Norway and
the Department of Microbiology
Faculty of Dentistry University of Oslo, Blindern, Oslo 3, Norway

Chlorpromazine Inhibition of *p*-Aminophenol Glucuronidation by Rat Hepatoma Cells in Culture

By

Erik Dybing

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Abstract. Chlorpromazine (0.01 mM to 0.15 mM) inhibits the glucuronidation of *p*-aminophenol and *p*-nitrophenol in a clonal strain of rat hepatoma cells, MH_1C_1, grown in culture, producing 50 % inhibition of *p*-aminophenol glucuronidation in a serumfree incubation medium at 0.07 mM. Chlorpromazine, at concentrations of 0.05 mM to 1.0 mM also inhibits the glucuronidation of *p*-aminophenol in homogenate prepared from the rat hepatoma cells (50 % inhibition at 0.25 mM) this inhibition was found to be non-competitive. The effect of chlorpromazine is enhanced when serum is excluded from the incubations.

Key-words: Chlorpromazine – membrane stabilization – drug metabolism – glucuronyl transferase – cell culture.

Chlopromazine (CPZ) is known to affect a multitude of biological membranes, exerting a biphasic action, with stabilization at low concentrations and increasing membrane permeability at higher concentrations. Excitable cells exposed to membrane stabilizers show a decrease in excitability (SHANES 1963). Erythrocytes can be protected against hypotonic, glycerol or urea induced lysis by CPZ (FREEMAN & SPIRTES 1963) Isolated rat liver lysosomes are stabilized against vitamin A or *E. coli* labilization (GUTH *et al.* 1965) and rat liver mitochondrial swelling as measured by water of sucrose entry is inhibited by CPZ (SPIRTES & GUTH 1963). CPZ decrease cardiac excitability contractility and potassium efflux in the isolated, perfused rat heart (LANGSLET 1970). At higher concentrations, however the phenothiazines cause lysis of red cells both *in vitro* (FREEMAN & SPIRTES 1963) and *in vivo* (AHTEE & PAASONEN 1965) leakage of liver enzymes from Chang cells in suspension (DUJOVNE & ZIMMERMANN 1969) into the medium is induced by CPZ.

The possible stabilizing effects of CPZ on the liver cell membrane could lead to a diminished cellular uptake of drugs and thus cause inhibition of drug metabolism. CPZ is known to affect drug metabolizing enzymes in a microsomal system the N-demethylation of ethylmorphine is inhibited in a competitive manner (RUBIN *et al.* 1964), indicating metabolism by the same hydroxylating enzyme. According to DE BARREIRO (1965) CPZ does not inhibit UDP-glucuronyl transferase (UDP-GT) in a homogenate system using *p*-nitrophenol (PNP) as substrate. Uridine diphosphate glucose dehydrogenase is non-competitively inhibited, however by a free radical formed from CPZ (LEVY & BURBRIDGE 1967)

Earlier reports have shown that the clonal rat hepatoma strain MH_1C_1 grown in culture, in addition to several liver specific functions (TASHJIAN JR. *et al* 1970) glucuronidates *p*-aminophenol (PAP DYBING & RUGSTAD 1972) and bilirubin (RUGSTAD *et al* 1970). This system which consists of living, multiplying cells with an intact outer membrane should prove suitable for studying the effects of membrane stabilizers on the glucuronidation process, and might circumvent some of the difficulties encountered in experiments with intact animals or in subcellular systems.

Material and Methods

Methods of cell culture

The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco's modified Eagle medium supplemented with 15 % horse serum and 2.5 % foetal calf serum and antibiotics as described previously (DYBING & RUGSTAD 1972) Cell protein was determined according to OYAMA & EAGLE (1956) using bovine serum albumin as standard.

Cell culture experiments

Replicate subcultures were incubated with substrates (to PAP 1-ascorbic acid was added in a final concentration of 1.0 mM in order to prevent auto-oxidation) in a serum-containing or serum-free medium, in a total volume of 10 ml for 4 hours at 37° The production of metabolites or the disappearance of substrates was assayed in aliquots of the incubation mediums at the desired time intervals.

Homogenat experiments.

The rat hepatoma cells in culture were removed with an icecold 0.02 % EDTA-barbital/NaCl-buffer pH 7 4 spun down and homogenized for 15 minutes with a glass-glass homogenizer in 1.0 ml of a 125 mM Tris-maleate buffer pH 7 4 The homogenate was centrifugated at 1,000 x g for 10 minutes at 4 and the supernate was used as a source of enzyme. In addition to 0.1 ml of the homogenate the incubation system consisted of the following constituents (final concentrations): PAP 0.10 to 0.25 mM UDPGA 4.0 mM Tris-maleate buffer 75 mM, pH 7 4 $MgCl_2$ 10.0 mM, and CPZ at various concentrations in a total volume of 0.5 ml. Glass-stoppered test-tubes were incubated for 30 minutes in a water shaking-bath at 37 the reaction was stopped with 0.5 ml icecold 0.5 M trichloracetic acid (TCA).

Table 1

Effect of CPZ on PAP-glucuronide formation and PNP disappearance in MH_1C_1 cell cultures in a serum-containing medium. 0.20 mM of substrates was incubated for 4 hours at 37° without or with CPZ at 0.10 and 0.15 mM respectively. Assays as in methods. Values are means ± S.D. Numbers in brackets refer to number of flasks.

Substrate	Substrate alone nmol/mg × hrs	With CPZ 0.10 mM nmol/mg × hrs	With CPZ 0.15 mM nmol/mg × hrs
p-Aminophenol	50.8 ± 2.3 (4)	39.5 ± 2.5 (4)	28.0 ± [illegible]3 (4)
p-Nitrophenol	67.1 ± 5.3 (4)	24.1 ± 4.1 (4)	18.4 ± 2.7 (4)

Drug assays.

The following assays were used: PAP-glucuronide formation (DYBING & RUGSTAD 1972), PAP disappearance (KATO & GILLETTE 1965), PNP (INSELBACHER *et al.* 1962), and CPZ and CPZ-sulfoxide (SALTZMAN & BRODIE 1956).

Pulse-labelling with ¹⁴C-alanine.

At the end of a cell culture experiment with PAP 0.20 mM and CPZ 0.10 mM in serum-containing medium, pulse-labelling with 1.05 µci ^{14}C-alanine (specific activity 156 mci/mmol) for 40 minutes was made. The cells were then removed with ice-cold 0.02 EDTA-barbital/NaCl-buffer pH 7.4 and precipitated with 10 % ice-cold TCA. After 30 minutes, the precipitate was spun down at 6000 r.p.m. for 10 minutes. The pellet was washed three times in the TCA-solution, twice in ethanol-diethyl-ether (2:1 v/v), and once in ethanol. The pellet was dissolved in 1 N-NaOH at 37° overnight. A 50 µl sample was heated together with 300 µl M hyamine 10-X in ethanol for 10 minutes at 70° then counted in 15 ml of scintillation fluid (100 g naphthalene, 5 g PPO 0.1 g POPOP in 400 ml 1.4-dioxane, 400 ml xylene, and 200 ml ethanol) in Packard Tri-Carb 3003 liquid scintillation spectrometer

Chemicals.

Para-aminophenol (PAP), para-nitrophenol (PNP), and chlorpromazine-HCl were obtained from Norsk Medisinaldepot (NMD). UDPGA (tri-ammonium salt), β-glucuronidase (type I, bacterial), and 1-ascorbic acid were purchased from the Sigma Company. ^{14}C-alanine through Institutt for Atomenergi, Kjeller from Amersham, England.

Results

From table 1 it is apparent that CPZ at concentrations of 0.10 and 0.15 mM inhibits the metabolism of the two subtrates PAP and PNP by the rat liver cell culture in a serum-containing incubation medium. PAP-glucuronide formation was measured directly. PNP metabolism was measured by substrate disappearance. 85 % of this substrate loss consists of PNP-glucuronide (using β-glucuronidase). It is also evident that in the cell culture system with serum present, the metabolism of PNP is inhibited to the greatest relative

The possible stabilizing effects of CPZ on the liver cell membrane could lead to a diminished cellular uptake of drugs and thus cause inhibition of drug metabolism. CPZ is known to affect drug metabolizing enzymes in a microsomal system: the N-demethylation of ethylmorphine is inhibited in a competitive manner (RUBIN *et al.* 1964), indicating metabolism by the same hydroxylating enzyme. According to DE BARREIRO (1965) CPZ does not inhibit UDP-glucuronyl transferase (UDP-GT) in a homogenate system using p-nitrophenol (PNP) as substrate. Uridine diphosphate glucose dehydrogenase is non-competitively inhibited, however, by a free radical formed from CPZ (LEVY & BURBRIDGE 1967).

Earlier reports have shown that the clonal rat hepatoma strain MH_1C_1 grown in culture, in addition to several liver specific functions (TASHJIAN JR. *et al.* 1970), glucuronidates p-aminophenol (PAP; DYBING & RUGSTAD 1972) and bilirubin (RUGSTAD *et al.* 1970). This system which consists of living, multiplying cells with an intact outer membrane should prove suitable for studying the effects of membrane stabilizers on the glucuronidation process, and might circumvent some of the difficulties encountered in experiments with intact animals or in subcellular systems.

Material and Methods

Methods of cell culture

The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco's modified Eagle medium supplemented with 15 % horse serum and 2.5 % foetal calf serum and antibiotics as described previously (DYBING & RUGSTAD 1972). Cell protein was determined according to OYAMA & EAGLE (1956) using bovine serum albumin as standard.

Cell culture experiments.

Replicate subcultures were incubated with substrates (to PAP 1-ascorbic acid was added in a final concentration of 1.0 mM in order to prevent auto-oxidation) in a serum-containing or serum-free medium, in a total volume of 10 ml for 4 hours at 37°. The production of metabolites or the disappearance of substrates was assayed in aliquots of the incubation mediums at the desired time intervals.

Homogenate experiments

The rat hepatoma cells in culture were removed with an icecold 0.02 % EDTA barbital/NaCl-buffer pH 7.4, spun down and homogenized for 15 minutes with a glass-glass homogenizer in 1.0 ml of a 125 mM Tris-maleate buffer pH 7.4. The homogenate was centrifugated at 1,000 x g for 10 minutes at 4° and the supernate was used as a source of enzyme. In addition to 0.1 ml of the homogenate the incubation system consisted of the following constituents (final concentrations): PAP 0.10 to 0.25 mM; UDPGA 4.0 mM; Tris-maleate buffer 75 mM pH 7.4; $MgCl_2$ 10.0 mM, and CPZ at various concentrations in a total volume of 0.5 ml. Glass-stoppered test-tubes were incubated for 30 minutes in a water shaking-bath at 37°; the reaction was stopped with 0.5 ml icecold 0.5 M trichloracetic acid (TCA).

1963 LANGSLET 1970), as are the LDR plots of other parameters in the isolated, perfused rat heart (LANGSLET 1970). One could argue, however since CPZ is concentrated intracellularly that the sole action represents an inhibition of the enzyme. The intracellular active concentration of CPZ is not known (about three-fold higher concentration as compared to that in the outer medium would be necessary to obtain inhibitory effects as explained by the intracellular action only)

CPZ is bound extensively to plasma albumin, 91–99 / by human plasma over a concentration range from 2.3×10^{-8} M to 4.3×10^{-4} M (CURRY 1970) The differences in potencies of CPZ shown in various systems might be explained when taking this into account. The inhibition of the overall glucuronidation of PAP is clearly shown to be diminished when serum is present. The results seen here without any serum at concentrations from 0.01 to 0 10 mM are in agreement with those concentrations found to stabilize erythrocytes against haemolysis (FREEMAN & SPIRTES 1963 SEEMAN & WEINSTEIN 1966) and those showing decreased cardiac excitability con tractility and potassium efflux in the isolated, perfused rat heart (LANGSLET 1970 no albumin in the perfusate) The result obtained when the cells were incubated in a serum-containing medium is perhaps more relevant to *in vivo* systems.

In the cell culture system a biphasic effect of CPZ was also noted. When the concentration of CPZ is raised, profound cellular effects are seen as symptoms of increased permeability The liver cells used begin to show visible toxic effects at 0.20 mM after 4 hours of incubation in a serum-containing medium, without serum these changes begin at 0.10 mM. Complete cell detachment is seen at 0.50 mM and 0 15 mM respectively Using cultured rat heart cell cultures (albumin-containing growth medium) FRIEDMAN *et al.* (1969) found intracellular vacuoles and detachment within 24 hours at a CPZ-concentration of 0 10 mM while destruction of the majority of the cells occurred after 5 minutes when CPZ at 1 0 was added to the medium. Concentrations of CPZ above 0 25 mM for 15 minutes caused leakage of lactate dehydrogenase, maleate dehydrogenase, and aspartate aminotransferase from Chang cells into the serum-free suspension medium (DUJOVNE & ZIMMERMANN 1969)

The effect of CPZ in the rat hepatoma culture system might be more pronounced as compared to similar effects in the normal intact liver where metabolic degradation of the inhibitor takes place. Indications of metabolic alteration of CPZ by the cell culture were not seen. This is in accordance with the low values or absence of drug-oxidizing activity found in various hepatomas (HART *et al.* 1965). A competition with PAP for the UDP-GT by a hydroxylated CPZ-metabolite should therefore not occur CPZ-sulfoxide, the third most prevalent metabolite in the rat (BERMAN & SPIRTES 1971), has

little membrane stabilizing effects of its own (SEEMAN & WEINSTEIN 1966). Differences in membrane integrity between cells in culture, hepatomas, and normal liver cells in the intact organ should also be taken into consideration.

The pulse labelling experiment might indicate that CPZ exerts a wide influence on the cellular uptake of low molecular substances by the liver. In the intact animal (mice) a single injection of 10 mg/kg CPZ causes enhanced incorporation of labelled glycine into liver proteins, however 1.0 mM CPZ inhibits protein synthesis in liver slices by approximately 50 / (KRAUS 1968). RAGHUPATHY *et al.* (1970) found little or no effect of CPZ (0.5 and 1.0 mM) on the incorporation of ^{14}C-phenylalanine into protein by rat liver ribosomes.

It is interesting to note that the effect of CPZ in this system suggest an inhibition of cellular uptake of different substances, whereas most other studies with CPZ have shown inhibition of membraneous effluxes.

Acknowledgements

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From the Institute of Pharmacology University of Oslo, Blindern, Oslo 3, Norway

Effect of Noradrenaline and Isoprenaline on the Circular and Longitudinal Muscle of the Oestrogen Dominated Rabbit Uterus

By

Britt Ingjerd Nesheim

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Abstract. Longitudinal and circular muscle strips from the oestrogen dominated rabbit uterus were stimulated *in vitro* with the α- and β-adrenergic stimulating drugs, noradrenaline and isoprenaline. The isometric contractions were recorded. Noradrenaline produced an increase in force and frequency of the contractions. No differences were found between the circular and the longitudinal muscle strips, or between the different parts of the uterus. Isoprenaline produced an inhibition of the spontaneous activity of the muscle strips. There was a difference between the response of the circular and the longitudinal muscle strips. The spontaneous activity of the longitudinal strips could be totally inhibited when isoprenaline was present in the organ bath in the concentration 2×10^{-7}M. The spontaneous activity of the circular strips could only be inhibited by approximately 50 % even with isoprenaline in a concentration of 2×10^{-6}M. Higher concentrations of isoprenaline had a stimulating effect. The ED50 of the circular strips was 60 times the ED50 of the longitudinal strips. No difference was found between the different parts of the uterus. No obvious alteration with regard to the sensitivity to the drugs was observed after cold storage for 24 hours.

Key-words: Uterus – receptors, drug.

In animals with a bipartate uterus, such as the rabbit, the muscular coat of the uterus has been shown to consist of an outer longitudinal and an inner circular layer The layers are separated by a well developed vascular plexus (SOBOTTA 1891). It has been established by several workers that the response of the uterine muscle to catecholamines is dependent on whether it is oestrogen or progesterone dominated (for references see MARSHALL 1970). Under oestrogen dominance the response of the rabbit uterus *in vivo* to α-adrenergic stimulation is a contraction, while the response to β-stimulation is inhibition of spontaneous motility (WILLEMS & DE SCHAEPDRYVER 1966). *In vitro* experiments on prepartum and postpartum rabbit uteri, have shown

striking differences in the response to adrenaline in different parts of the uterus, and differences have also been found in the responses of the longitudinal and circular muscle strips. The cervical segments were contracted more easily by adrenaline than the tubal and placental segments, while the circular strips responded more frequently with augmentation of activity than the longitudinal strips. (BONNYCASTLE & FERGUSON 1941) Similar experiments on non-pregnant uteri have not been performed.

The present study was designed to test whether there was any difference in the responses of the different parts of the uterus, or between the longitudinal and circular muscle layer following adrenergic α and β-stimulation in the oestrogen dominated rabbit uterus.

Materials and methods

Fifteen rabbits weighing 2–3 kg were used for the experiments. Ovarectomy was performed through an incision of about 2 cm in the flanks, after which tablet of 25 mg diethylstilboestrol was implanted subcutaneously. One week later the animal was given a blow on the neck and bled to death by cutting the carotid arteries. The uterus was immediately removed and placed in modified Ringer' solution (NaCl 120.35 KCl 5.91, $NaHCO_3$ 15.42, $MgCl_2$ $6H_2O$ 1 19 NaH_2PO_4 $2H_2O$ 1.18, $CaCl_2$ $2H_2O$ 2.35 glucose 11.48 mM/1). The pH of the solution was 7 4. The same preparation was used on two consecutive days, i. e. fresh, and after having been stored overnight at 4 There was no obvious alteration in the responses after 24 hours in cold storage.

For each experiment 4 muscle strips were cut from the uterus, 2 longitudinal ones from each other so the strips were just cut from the uterus in the desired direction. The 1 1.5 cm long. It was impossible to dissect the longitudinal and circular muscle layers from each other so the strips were just cut from the uterus in the desired direction. The small diameter of the strips probably makes it possible to ignore the fibres running in the *transverse direction. This has been shown to be the case in strips from large veins* by MCCALDEN *et al.* (personal communication 1970). The muscle strips were placed in two baths containing 50 ml of the same modified Ringer' solution, kept at 41 The baths were connected to reservoir and washed out by prewarmed solution. The baths were oxygenated with 5 % CO_2 in O_2. Each bath contained one longitudinal and one circular strip.

The lower end of the strip was tied by surgical silk to glass rod, the upper end to Grass force-displacement transducer FT03C, the contractions thus being isometric. The transducer was connected t a Grass polygraph, which recorded the contractions with ink on paper The strips were stretched t mean tension of 1 g. They were then allowed to remain in the bath for at least one hour before any addition of drugs. After the addition of each drug the bath was washed out twice. The next addition of drug was not made until t least five minutes had passed, and in case of isoprenaline, not until the preparation had reached stable spontaneous activity The time passing from washing out until new addition of drug, was usually approximately 15 min.

The drugs used were noradrenaline and isoprenaline, phentolamine (regitin®) and phenoxybenzamine (benzylytum NFN).

Results

Noradrenaline (NA)

The typical response to NA was an increase in the frequency as well as in the force of the contractions. With increasing doses an increase in tone was also observed, but this usually subsided in 2–3 min. (fig. 1) The longest period of observation of the NA-evoked contractions before washing out, was 20 min 50 muscle strips from 9 rabbits were tested with NA in the dose range $2 \times 10^{-8} - 2 \times 10^{-4}$M. The response to the same dose of NA varied in the different preparations, the threshold dose varying from 2×10^{-8} to 2×10^{-7}M.

In 104 drug additions to pairs of strips consisting of one circular and one longitudinal strip the responses were approximately equal in 81 cases, the circular strips giving a greater response in 10 cases and the longitudinal strips giving a greater response in 13 cases. No difference was found between the tubal end, the middle and the cervial end of the uterus The response to NA was blocked by phentolamine 5×10^{-6}M.

Isoprenaline (IP)

IP caused an inhibition of the spontaneous contractions of the muscle strips. For tests of this type, only those muscle strips with a fairly regular spontaneous activity were used.

The typical response to IP was a decrease in the force of the contractions. A decrease in frequency was also sometimes observed. With very large doses, however ($2 \times 10^{-6} - 2 \times 10^{-4}$M) a stimulation was achieved. This stimulation was completely blocked by the α-blocking agent phenoxybenzamine 2×10^{-6}M

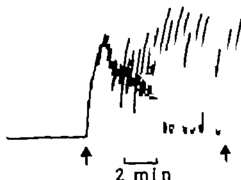


Fig. 1. Response to NA 2×10^{-7}M of a longitudinally cut muscle strip from the cervical end of the oestrogen dominated rabbit uterus. Ordinate: force during isometric contractions. Abscissa: time. NA is added at the first arrow and washed out at the second arrow There was no spontaneous activity in this preparation.

No differences were found between the responses of the tubal end, the middle and the cervical end of the uterus, but a great difference was found between the responses of the circular and the longitudinal muscle strips. With the same dose of IP the longitudinal muscle showed a much greater inhibition than the circular muscle. A typical response from a circular and a longitudinal muscle strip is shown in fig. 2.

In order to obtain a dose-response curve for the circular and the longitudinal muscle strips, 6 doses of IP were used, 2×10^{-6}M 2×10^{-7}M, 2×10^{-8}M, 2×10^{-9}M, 2×10^{-10}M and 2×10^{-11}M. A measure of the degree of inhibition was obtained by measuring the height of each contraction during one 5 min. interval just before, and another 5 min. interval after the addition of the drug. The heights of the contractions in each interval were added together The first minute after the addition of the drug was ignored, in order to allow the drug to produce its effect. The difference between these two sums as percentage of the sum of the height of the contractions before the addition of the drug, was used as a measure of the inhibition. The responses to a certain dose in one preparation varied a great deal from one addition of the drug to another and in a typical experiment the highest and lowest values obtained for the inhibitions could deviate by

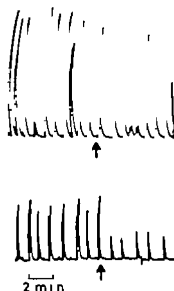


Fig. 2. A fairly representative response to β-stimulation of a circular (upper tracing) and longitudinal (lower tracing) muscl strip. Spontaneous contractions. Ordinate: force during isometric contractions. Abscissa: time. At the arrow IP 2×10^{-9}M was added. The contractions of the circular and longitudinal muscle strip were reduced by approximately 10 and 50 % respectively

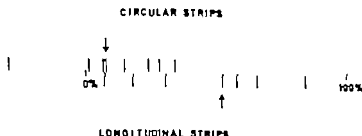


Fig. 3 The inhibitory effect on circular and longitudinal muscle strips of IP 2×10^{-9}M. Each vertical line represents the median of the inhibitions for this dose of the drug in one rabbit. Circular strips on the upper side of the line, longitudinal strips on the lower side. 0 % and 100 % inhibition are marked. The position of the median of these medians is indicated by arrows.

30 % from the median. The median of the inhibitions for each dose of the drug for the longitudinal and the circular muscle strips was calculated in each rabbit, each of these medians representing on an average 3–4 additions of the drug. For each dose such medians obtained from the different rabbits varied considerably. An example is shown in fig. 3. Each vertical line represents the median from one rabbit. The median of these median inhibitions from the different rabbits was then found (arrows in fig. 3). The latter medians are plotted in a dose-response curve in fig. 4. Each point in fig. 4 represents on an average 17 additions of the drug. The differences between the inhibitions obtained in the longitudinal and circular strips were shown to be significant according to a Wilcoxon's two sample test. For the dose 2×10^{-6}M $\alpha = 0.0010$ for 2×10^{-7}M $\alpha = 0.0039$ for 2×10^{-8}M $\alpha = 0.0002$, for 2×10^{-9}M $\alpha = 0.0144$. For the doses 2×10^{-10}M and 2×10^{-11}M, the differences were not significant ($\alpha = 0.5000$ and 0.3429 respectively).

In some of the preparations the spontaneous activity consisted of alternating stronger and weaker contractions. By the addition of IP the strong contractions were reduced to the level of the weaker ones, but these could not be inhibited, even with the largest doses of IP. The frequency of the contractions was not reduced. Fig. 5 is an illustration of this phenomenon.

Discussion

This investigation confirms that isoprenaline inhibits the spontaneous activity of isolated muscle strips from the uterus of the oestrogen dominated rabbit as found previously by WILLEMS & DE SCHAEPDRYVER (1966). The experiments conclusively show that the longitudinal muscle strips are much more sensitive to isoprenaline in the dose range 2×10^{-9}M – 2×10^{-6}M

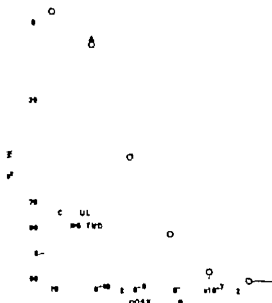


Fig. 4. Log dose-response curves of the circular and the longitudinal muscle strips to IP. Abscissa, doses in mol. Ordinate: inhibition as per cent of the preceding activity (see text).

than the circular strips. No such difference was found for the responses to noradrenaline, which consisted of increases in the activity and the tone of the muscle strips. Nor was any difference found between the cervical end,

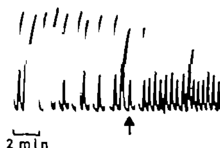


Fig. 5. The response of one circul r muscl strip to the addition of IP 2×10^{-9}M. Ordinate: force during isometric contractions. Abscissa, time. Before addition of the drug, the activity is seen to consist of alternating stronger and weaker contractions. After addition of the drug, the stronger contractions are reduced to the level of the ones. The strip is taken from the cervical end of the uterus.

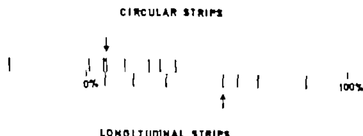


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Another explanation for the different responses could be a difference in the unknown chain of events linking the receptor stimulus to the biological effect, in this case the reduction of spontaneous contractions.

Electrophysiologically it has been shown (MARSHALL 1969) that in the oestrogen treated rat uterus there are definite pacemaker areas located at the cervical or the ovarian end of the uterus. In the present investigation some of the preparations showed alternating stronger and weaker contractions. On addition of isoprenaline the stronger contractions were reduced to the level of the weaker ones, the rate of the contractions not being reduced. The height of the contractions could not be reduced further even with the highest doses of isoprenaline. An explanation of this observation could be that the weaker contractions were the results of pacemaker activity. Isoprenaline could then have reduced the spread of contractions through the tissue, but not the pacemaker activity itself. This interpretation, however is in conflict with the ideas of BÜLBRING & TOMITA (1969) who have shown that in the guinea-pig taenia coli isoprenaline suppresses the spontaneous spike generation.

It is difficult to see any immediate physiological implications of the different sensitivities of the longitudinal and the circular muscle fibres to β-stimulation. There are however reasons for speculation about such implications. The circular muscle fibres are the "real" uterine muscle, developed from the Müllerian ducts, while the longitudinal muscle fibres are derived from the serosa (SOBOTTA 1891). It is therefore possible that the two muscle layers might have different characteristics and different functions during implantation and parturition.

The rabbit uterus may be oestrogen-dominated during the earliest part of blastocyst transport, but very soon it becomes strongly progesterone-dominated, so that the blastocyst transport and implantation take place in a strongly progesterone-dominated uterus (BÖVING 1963). Approximately 24 hours before parturition the hormone dominance changes over so that parturition takes place in an oestrogen dominated uterus (SCHOFIELD 1957).

The circular and longitudinal muscle fibres must be assumed to have different functions during parturition, the circular muscle being responsible for the peristalsis bringing the foetus towards the cervix, and the longitudinal muscle for shortening the uterus to let the foetus into the vagina. It is difficult to see any obvious functions of the different responses to β-stimulation of the circular and longitudinal muscle fibres during parturition.

The results presented call for the same type of experiments to be carried out on the progesterone dominated rabbit uterus. One would like to know if the difference between the longitudinal and circular muscle is a general characteristic of uterine muscle, or whether it varies with hormone dominance. These findings will, however not lead to considerations relevant to the situation in the human uterus, as the whole uterus in women is phylogenetic

ally derived from the Müllerian ducts, as is the circular muscle in the rabbits. The longitudinal muscle from the serosa is not developed in humans (SOBOTTA 1891)

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From the Oakdale Toxicology Center Department of Pharmacology College of Medicine, University of Iowa, Iowa City Iowa 52240, U.S.A.

The Effects of Maternally–administered Morphine on Rat Foetal Development and Resultant Tolerance to the Analgesic Effect of Morphine

By

Torkell Jóhannesson and Bernard A. Becker

(Received January 14, 1972 Accepted March 17 1972)

Abstract: Groups of 6–9 pregnant (sperm = Day 1) Sprague-Dawley (Simonsen) rats were injected with morphine sulphate, 20 mg/kg subcutaneously on gestational days 2–5, 7–9 or 11 13. Pups delivered by Caesarian section on gestational day 22 were essentially free of any observable teratogenic changes including bone defects. However offspring of morphine-treated rats (20 mg/kg/day subcutaneously on gestational days 17 18, 19 and 20) which were allowed to litter normally exhibited on day 12 13 significantly ($P < 0.05$) decreased response to a nociceptive (55 surface) stimulus 30 min. following an injection of morphine sulphate 0.45 mg/kg subcutaneously (offspring of morphine-treated mothers, 16.8 sec. ± 2.3 S.E.M. versus control offspring, 25.2 sec. ± 1.7 S.E.M.). On days 21–22 of age larger doses of morphine were required to elicit an analgesic response in the young. Following an injection of morphine sulphate, 10 mg/kg subcutaneously the maternal animals also showed significant reduction in reaction time 30 minutes later (treated, 7.6 sec. ± 0.6 S.E.M versus control, 16.1 sec. ± 0.6 S.E.M.) and 60 minutes later (treated, 7.5 sec. ± 0.9 S.E.M. versus control, 20.8 sec. ± 2.9 S.E.M.) as compared to paired waterinjected control pregnant animals.

Key-words: Morphine – pregnancy – placental transfer malformation – teratology – birth defects – neonatal rats – analgesia – tolerance.

In the available literature there seem to be no reports of any possible teratogenic effects of morphine in rats. In mice, on the other hand, HARPEL & GAUTIERI (1968) reported that morphine produces minor teratogenic effects *in very high doses. Only* NICÁK & MASNYK *(1966)* seem to have investigated the analgesic action of morphine in young rats. Apparently no one has studied the *in utero* development of tolerance to this action of morphine although there is ample evidence which indicates that morphine like other

narcotic analgesics passes through the placenta into the foetal circulation (cf. SANNER & WOODS 1965). The experiments reported here were, therefore, performed in order to investigate whether morphine administered to rats at various time periods during pregnancy might affect the development of the foetuses or induce tolerance of the resulting neonates to the analgesic action. For comparison, the maternal rats were also studied to see whether the morphine doses given were effective in inducing tolerance to the analgesic action of morphine.

Methods

Animals.

Adult female Sprague-Dawley (Simonsen) rats, around 200 g body weight, were housed in stainless steel cages and maintained in constant environmental conditions with free access to a commercial food preparation (Wayne Lab-Blox, Allied Mills, Inc.) and tap water. The rats were placed with males of known fertility of the same stock, using one male for two females, from approximately 4:00 p.m. to 8:00 a.m. on the following day. The finding of sperm in vaginal smears was taken as a sign of copulation, this time being designated as the first day (day 1) of pregnancy. About one-fifth of the rats did not conceive and were eventually excluded from the experiments. The pregnant rats were kept in groups of three or four until day 22 when they were killed and the foetuses removed for examination. Some rats which were allowed to deliver naturally were separated on day 21 and housed singly in cages furnished with pans covered with ground corn cob bedding. The studies described were conducted during July, August, September and October, 1971.

Treatments.

Morphine sulphate, U.S.P. was dissolved in distilled water and injected subcutaneously in amounts of 10 or 20 mg/kg in a volume of 2 ml/kg (adult animals) or 0.45, 0.625 or 1.25 mg/kg in 10 ml/kg (young animals). In the adult animals injections were made in the middle of the back at the midline. In the young animals, injections were made in the neck at the midline, using a short 25 gauge needle. The doses quoted refer to morphine as the free base. Table 1 shows the various treatment groups.

Teratology studies.

Three experiments were performed using six groups, each of 10–11 rats. *Group I* received morphine, 20 mg/kg, on days 2, 3, 4 and 5. *Group II* on days 7, 8 and 9; and *Group III* on days 11, 12 and 13 of pregnancy. *Groups IV, V* and *VI* consisted of the respective control animals which were injected with water (2 ml/kg, subcutaneously).

The animals were killed by excess carbon dioxide and the foetuses removed by Caesarean section. The number of living foetuses and implants was noted. The foetuses were examined for external anomalies, weighed, and measured as previously described (GIBSON & BECKER 1968, HARRISON & BECKER 1969).

Half the number of the foetuses were fixed for two weeks in Bouin's solution, then sectioned by hand and examined under a dissecting microscope for soft tissue anomalies as described by WILSON (1965). The other half of the foetuses were fixed in denatured ethanol for 1 month, skinned, eviscerated and cleared in 1 % (w/v) potassium hydroxide for five days and thereafter stained with Alizarin red-S. Stained skeletons were examined and measured under a dissecting microscope fitted with a reticle. Long bone measurements were recorded in reticle units (1 reticle unit equal to 0.14 mm).

Table I
Experimental groups

"Adult" means the maternal Sprague-Dawley rats except for Group IX which consisted of mature, non-pregnant rats. "Young" are the offspring of the correspondingly numbered "Adult" group. "Dosage" refers to treatment of maternal animals during pregnancy. "Size" indicates the final number of animals in each group. Dosages are expressed as the free morphine base given subcutaneously.

Study	Group Number	Group Size	Age	Dosage mg/kg	Day Treatment	Day Experiment
Teratology	I	7	Adult	20	G 2–5[1]	G 22[1]
	II	6	Adult	20	G 7–9	G 22
	III	7	Adult	20	G 11–13	G 22
	IV	8	Adult	0	G 2–5	G 22
	V	7	Adult	0	G 7–9	G 22
	VI	9	Adult	0	G 11–13	G 22
Tolerance to Analgesia	VII	14	Adult	20	G 17–20[1]	28[1) 2)]
	VIII	13	Adult	0	G 17–20	28
	IX	10	Adult	0	none	28
	VIIa	20	Young	Treated	G 17–20	12–13
	VIIIa	19	Young	0	G 17–20	12–13
	VIIb	10	Young	Treated	G 17–20	20–21
	VIIIb	10	Young	0	G 17–20	20–21

1) The symbol "G" means gestational. Numbers not preceded by this symbol denote days after birth of the young.
2) These animals received a challenge dose of morphine on the experimental day indicated, adults, 10 mg/kg; 12 day old animals, 0.45 mg/kg; and 20 day old animals, 0.625 or 1.25 mg/kg.

Analgesimetry

The analgesic action of morphine in maternal and young rats of at least three weeks of age was determined using the hot-plate method as described by JÓHANNESSON & WOODS (1964). The animals were tested on the hot-plate 30 minutes and just before injection and then 30 minutes and 60 minutes after injection.

The technique mentioned above cannot be successfully applied to animals younger than three weeks old, since rats that are younger than approximately 17 days usually neither lick their paws nor try to jump from the surface of the hot plate, i. e. the usual end-points. In experiments with 12–13 day old rats the following modified procedure was used. The animals were placed on their four feet on the surface of the hot-plate, using a 600 ml beaker with the bottom removed as a restraining device. *Pushing and driving of the nose against, or attempts to climb the inner surface of the cylinder was taken as the end-point.* In our hands this technique has been found sufficiently reproducible to allow comparison of analgesic responses in rats of this age. The time sequence of testing on the hot-plate was the same as in experiments on older

The degree of analgesia of morphine is expressed as the mean reaction time in seconds for a group of animals, i.e. the mean time in seconds which elapsed from the moment each animal was placed on the hot-plate until it reacted to the heat. The animals were taken off the plate if they did not react in 30 seconds. Thus 30 seconds is the highest score given.

Analgesic studies T lerance

Two groups of maternal rats were used. *Group VII* (14 pregnant rats) received morphine, 20 mg/kg subcutaneously on gestational days 17 18, 19 and 20. *Group VIII* (13 control pregnant rats) were injected with w ter 2 ml/kg subcutaneously on the same days. The experiments were performed in two parts, using about one-half of the animals each time with a month interval. This was done in order to corroborate the results of those first experiments.

Table 2

Number of implants and foetuses and foetal weights, lengths and widths as observed following delivery of maternal rats by Caesarean section on day 22. The weights of the maternal rats are also included. All values are mean values ± S.E.M. Numbers of implants and measured foetuses refer to means per litter in each group. Rats in Groups I, II and III were treated with morphine, 20 mg/kg subcutaneously during pregnancy whereas Groups IV V and VI consisted of the respective control animals.

Numbers, measurements and weights	Rats treated on days 2, 3 4, 5		Rats treated on days 7 8, 9		Rats treated on days 11, 12, 13	
	Group I (N=7)	Group IV (N=8)	Group II (N=6)	Group V (N=7)	Group III (N=7)	Group VI (N=9)
No. implants	11.9 ± 1.2	13.5 ± 0.5	11.0 ± 1.1	9.4 ± 1.6	12.6 ± 0.5	11.6 ± 1.3
No. meas. foetuses	10.9 ± 1.8	12.6 ± 0.9	10.8 ± 1.1	9.3 ± 1.6	12.4 ± 0.4	11.5 ± 1.3
Foetal weight (g)	5.5 ± 0.1	5.2 ± 0.1	5.0 ± 0.1[b]	5.6 ± 0.2	5.1 ± 0.1	5.3 ± 0.2
Crown-rump dist. (mm)	42.0 ± 0.4	41.1 ± 0.2	41.6 ± 0.2	43.2 ± 0.6	42.1 ± 0.3	41.2 ± 0.4
Trans-umbil. dist. (mm)	12.0 ± 0.9	11.9 ± 0.1	11.8 ± 0.1	12.1 ± 0.2	12.0 ± 0.1	12.2 ± 0.2
Maternal Weight (g)	393±10.6	395±16.7	372±15.8	358±12.1	356±12.1	373±13.0

Significantly different ($P < 0.05$) from Group IV See text.

[b] Significantly different ($P < 0.05$) from Group V See text.

Within 24 hours (8–24 hours) after natural delivery the young in each litter were examined for external anomalies, counted and weighed. Each litter was standardized to eight pups (4 male and 4 female) when ever possible. At the age of 12 13 days a number of animals were taken out of each litter injected with morphine, 0.45 mg/kg subcutaneously (Groups VIIa and VIIIa) and tested for the degree of analgesia. The remaining young were similarly used for nalgesic experiments at the time of weaning when 20–21 days old (Groups VIIb and VIIIb). The dose of morphine was 0 625 or 1.25 mg/kg subcutaneously in these experiments. Preliminary experiments indicated that tolerance to the analgesic effect of morphine was of a longer duration in the maternal rats than in the young. The maternal animals which had been treated with either morphine or water on gestational days 17 18, 19 and 20 (Groups VII and VIII) were therefore tested four weeks fter delivery along with a group of 10 control adult, non-pregnant female rats. (Group IX). All three groups were injected with morphine sulphate, 10 mg/kg subcutaneously and tested as described above.

Statistical analyses were preformed by the Mann Whitney "U" test and student's grouped "t test. (GOLDSTEIN 1964). The level of significance was taken as $P \leq 0.05$.

Results

Teratology studies.

Lethality Six maternal rats died following the injection of morphine. Morphine was thus lethal in about 10 / of the adult animals following the first injection of morphine. No deaths were observed in the control groups.

The number of measured (viable) foetuses and implants per litter was almost the same in each pair of groups. The difference in the number of implants was not statistically significant within groups using the two sample rank test (table 2).

Body weights and measurements. Body weights of maternal rats on day 22, and foetal weights, lengths (crown-rump distance) and widths (transumbilical diameter) are shown in table 2. The differences observed were, on the whole, relatively small and could not be ascribed to the administration of morphine. Thus, the foetal weights were found significantly higher in Group V than in Group II whereas the foetal weights in Group I were significantly higher than in Group IV and no significant difference was found between the foetal weights in Groups III and VI.

Gross anomalies were not observed. Soft tissue anomalies were seen in three foetuses. hydronephrosis in a foetus from a morphine-treated mother· diaphragmatic hernia and situs inversus of the heart, respectively in two foetuses from mothers in the control groups.

Bones Except for some cases of incomplete ossification, mainly seen in the vertebrae and sternebrae of rats in all groups, no skeletal anomalies were observed. The femur tibia, fibula, humerus, radius and ulna were measured on both sides. The slight differences observed were insignificant and could not be ascribed to the administration of morphine.

Table 3

The analgesic action of morphine in maternal and non-pregnant female rats (10 mg/kg, subcutaneously) in unweaned rats 12–13 days old (0.45 mg/kg, subcutaneously) and 20–21 days old (0.625 mg/kg, subcutaneously). The maternal rats in *Group VII (morphine treated)* and *Group VIII (control animals)* were tested *four* weeks after delivery. The analgesic action is shown as the mean reaction time in seconds ± S.E.M. at 30 and 60 min. (Reaction times 3 and 4) after the injection of morphine. Included are the body weights of the animals and their reaction times 30 min. and just before the injection of morphine (Reaction times 1 and 2).

Reaction times and body weight	Maternal Group VII	Maternal Group VIII	12–13 days Old Group VII	12–13 days Old Group VIII[a]	20–21 days Old[a]	Non-pregnant females Group IX
	(N=14)	(N=13)	(N=20)	(N=19)	(N=10)	(N=10)
Reaction Time Time 1	5.5 ± 0.4	5.8 ± 0.6	5.9 ± 0.4	5.0 ± 0.3	6.4 ± 0.7	5.4 ± 0.4
Reaction Time 2	5.6 ± 0.4	5.4 ± 0.4	5.5 ± 0.5	4.5 ± 0.4	4.5 ± 0.5	5.4 ± 0.5
Reaction Time 3	7.6 ± 0.6[b]	16.1 ± 0.6	16.8 ± 2.3[b]	25.2 ± 1.6	16.7 ± 1.6	17.1 ± 2.3
Reaction Time 4	7.5 ± 0.9[b]	20.8 ± 2.9	16.6 ± 1.9	21.6 ± 1.8	10.5 ± 1.2	19.6 ± 2.9
Weight (g)	296 ± 20.3	301 ± 26.9	24.6 ± 0.9[b]	27.3 ± 0.8	32.3 ± 1.0	227 ± 11.0

[a] Randomly selected with regard to sex from unstandardized ($n > 8$) litters from untreated dams.

[b] Significantly less than corresponding control.

Analgesimetry. The analgesic responses (table 3) of rats in Group VII
1 (maternal rats treated with morphine on gestational days 17–20) at 30 and 60 minutes after the injection were obviously significantly less than those of the control animals (Group VIII). The analgesic action of morphine in the latter group was, on the other hand, very similar to that observed in a group of 10 non-pregnant, adult female rats which received the same dose of morphine (Group IX).

Fourteen rats in Group VII and thirteen rats in Group VIII littered. The average number of young per litter was 10.5 ± 0.7 (S.E.M.) in Group VII and 11.5 ± 0.9 (S.E.M.) in Group VIII. The mean weight of the total number of young in each litter was 71.9 ± 4.7 (S.E.M.) g and 68.0 ± 7.5 (S.E.M.) g in these groups, respectively. The slightly higher numbers of

young per litter in Group VIII was matched by a slightly lower body weight. These differences were not statistically significant.

The analgesic action of morphine (reaction times 3 and 4) in the 12–13 day old rats in Group VIIIa (offspring of the control animals) was higher than those of rats in Group VIIa (offspring of the morphine-treated animals). It was significantly lower in Group VIIa than in Group VIIIa at reaction time 3 but not 4. The mean body weights of young from morphine-treated rats (Groups VIIa) was significantly smaller than those of young from mothers which did not receive morphine (Group VIIIa).

In preliminary experiments, groups of 20–21 day old unweaned rats, taken at random from several unstandardized litters (i.e. not reduced to eight pups) derived from non-treated dams and having an average body weight of 30–35 g, were found to be less sensitive to the analgesic action of morphine than were the 12–13 day old rats. A dose of 0.625 mg/kg subcutaneously was required to give an appreciable degree of analgesia in these animals. The results of one such experiment are shown in table 3 (column 5). When the dose was increased to 1.25 mg/kg subcutaneously the animals usually responded with a maximal analgesic reaction (response time > 30 seconds).

At the age of 20–21 days, the rats in Groups VIIb and VIIIb weighed on an average 41 and 49 g; these values being significantly different. Most of them were injected with morphine, 0.625 mg/kg subcutaneously and showed an almost negligible analgesic response. The few animals then at hand were injected with morphine, 1.25 mg/kg subcutaneously and showed a moderate degree of analgesia or much less than that previously obtained in the above-mentioned 20–21 day old rats after 0.625 mg/kg subcutaneously.

Discussion and Conclusions

The lethal action of subcutaneous morphine in rats may be irregular and complex, so that the administration of different doses can result in about the same degree of lethality (JÓHANNESSON 1965). The dose used (20 mg/kg subcutaneously) in the investigation reported here is about the lowest which has been found to have a lethal effect in rats of both sexes generally used in this laboratory. Thus, even though no teratogenic or embryocidal effects were observed following the administration of the aforementioned dose of morphine (tables 1 and 2 and text), it must still be considered to be of such an order of magnitude as to be in the so-called teratogenic zone (WILSON 1965) since the dose resulted in the death of approximately 10 % of the treated females. The teratogenic effect of morphine in this species is therefore probably nil or very low. This is in agreement with the work of HARPEL & GAUTIERI (1968) in experiments on mice. These authors found that certain anomalies (exencephaly and axial skeletal fusions) occurred when morphine

Table 3

The analgesic action of morphine in maternal and non-pregnant female rats (10 mg/kg, subcutaneously)) in unweaned rats 12–13 days old (0.45 mg/kg, subcutaneously) and 20–21 days old (0.625 mg/kg, subcutaneously). The maternal rats in Group VII (morphine treated) and Group VIII (control animals) were tested four weeks after delivery. The analgesic action is shown as the mean reaction time in seconds ± S.E.M. at 30 and 60 min. (Reaction times 3 and 4) after the injection of morphine. Included are the body weights of the animals and their reaction times 30 min. and just before the injection of morphine (Reaction times 1 and 2).

Reaction times and body weight	Maternal Group VII	Maternal Group VIII	12–13 days[a] Old Group VII_A	12–13 days Old Group VIII	20–21 days Old[a]	Non-pregnant females Group IX
	(N=14)	(N=13)	(N=20)	(N=19)	(N=10)	(N=10)
Reaction Time Time 1	5.5 ± 0.4	5.8 ± 0.6	5.9 ± 0.4	5.0 ± 0.3	6.4 ± 0.7	5.4 ± 0.4
Reaction Time 2	5.6 ± 0.4	5.4 ± 0.4	5.5 ± 0.5	4.5 ± 0.4	4.5 ± 0.5	5.4 ± 0.5
Reaction Time 3	7.6 ± 0.6[b]	16.1 ± 0.6	16.8 ± 2.3[b]	15.2 ± 1.6	16.7 ± 1.6	17.1 ± 2.3
Reaction Time 4	7.5 ± 0.9[b]	20.8 ± 2.9	16.6 ± 1.9	21.6 ± 1.8	10.5 ± 1.2	19.6 ± 2.9
Weight (g)	296 ± 20.3	301 ± 26.9	24.6 ± 0.9[b]	27.3 ± 0.8	32.3 ± 1.0	227 ± 11.0

[a] Randomly selected with regard to sex from unstandardized ($n > 8$) litters from untreated dams.
[b] Significantly less than corresponding control.

Analgesimetry The analgesic responses (table 3) of rats in Group VII (maternal rats treated with morphine on gestational days 17–20) at 30 and 60 minutes after the injection were obviously significantly less than those of the control animals (Group VIII). The analgesic action of morphine in the latter group was, on the other hand, very similar to that observed in a group of 10 non-pregnant, adult female rats which received the same dose of morphine (Group IX).

Fourteen rats in Group VII and thirteen rats in Group VIII littered. The average number of young per litter was 10.5 ± 0.7 (S.E.M.) in Group VII and 11.5 ± 0.9 (S.E.M.) in Group VIII. The mean weight of the total number of young in each litter was 71.9 ± 4.7 (S.E.M.) g and 68.0 ± 7.5 (S.E.M.) g in these groups, respectively. The slightly higher numbers of

mothers at hand to use as a reference (cf the experiments with the adult animals) Further studies are needed in order to elucidate whether tolerance to the analgesic action of morphine induced *in utero* is of a similar magnitude and duration as that induced in the maternal rats. In this connection it would be of particular interest to investigate whether any changes in tolerance to the analgesic action of morphine in the young animals would correspond to changes which might occur during the same period in the sensitivity to this action of morphine.

There are two significant conclusions which may be drawn from this work. Firstly multiple (3 or 4 day) administrations of morphine to pregnant rats during pre-implantation, organogenesis or in the late growth periods of pregnancy do not result in obvious teratogenic changes. Secondly administration of morphine during late pregnancy results in tolerance to the analgesic effect of morphine which is clearly demonstrable 12 or 13 days after birth.

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From the Psychopharmacology Research Unit, Aarhus University Psychiatric Institute, State Mental Hospital 8240 Risskov Denmark (Chiefs: M. Schou and A. Amdisen)

The Relation Between Vasopressin Stimulation of Renal Adenyl Cyclase and Lithium-Induced Polyuria in Rats

By

Olaf Wraae Arne Geisler and Ole Vendelin Olesen

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Abstract. Renal adenyl cyclase activity was estimated in 3 groups of 14 rats. In each group 7 rats were given lithium-containing food and 7 served as controls. The first group was treated up to one week before the adenyl cyclase was measured. Group 2 was analyzed after 3 weeks of lithium-treatment when a polyuria had developed. In group 3 lithium-treatment was stopped after 5 weeks and adenyl cyclase measured one week later when the polyuria had disappeared. The serum and kidney lithium levels were the same in groups 1 and 2, but in the polyuric rats vasopressin stimulation was lower. After an ATP regeneration system had been added to the incubate the same phenomenon was seen. In group 3 the effect of vasopressin stimulation on adenyl cyclase was the same as in controls. Our results show that the lithium-induced inhibition of vasopressin-stimulated adenyl cyclase activity develops gradually is reversible and is independent of the lithium ion itself

Key-words. Adenyl cyclase – lithium – vasopressin – polyuria.

In a previous paper we compared the renal adenyl cyclase activity of rats with lithium induced polyuria with the activity of control rats not given lithium (GEISLER *et al* 1972). It was found that the formation of adenosine 3′ 5′ monophosphate (cyclic AMP) from ATP was stimulated less by vasopressin in homogenates from polyuric rats than in homogenates from rats not given lithium.

Both in man and in the rat the lithium-induced polyuria develops gradually: when lithium is discontinued, it disappears gradually. The aim of the present study was to determine the relation between the development and disappearance of polyuria and the response of the renal adenyl cyclase to vasopressin.

Three groups of rats were compared. They were all given lithium in doses which according to experience would lead to polyuria in three to four weeks (THOMSEN 1970). The first group was examined a few days after the start of the lithium administration when the serum and renal lithium levels had become stabilized but when polyuria had not yet developed. The second group was studied after the development of polyuria and the third group was studied after the discontinuation of lithium at a time when the polyuria had disappeared.

Material and methods

Male Wistar rats initially weighing about 200 g were randomly divided into three groups of 14 animals. In each group one half of the animals were given food containing lithium chloride in a concentration of 40 mM/kg dry weight; the other half received the same food without lithium. All the animals had free access to tap water. Group no. 1 was studied after lithium administration for 2–7 days: at this time the rats had not yet developed polyuria but their serum and kidney lithium levels were the same as in group no. 2. In group no. 2 the rats were examined after lithium administration for 3–4 weeks when a pronounced polyuria had developed. In group no. 3 lithium was discontinued after 5 weeks; the animals were studied about a week later when the polyuria had disappeared.

The determination of renal adenyl cyclase activity was carried out as described previously (GEISLER *et al.* 1972); a lithium treated rat and a control rat were always studied on the same day. In group 2 additional determinations of adenyl cyclase activity were carried out with a method involving the use of an ATP regeneration system. The final concentration in the incubate was phosphoenolpyruvic tricyclohexylammonium salt 0.6 mM and pyruvate kinase 12 µg/ml. The serum and kidney lithium concentrations were determined by flame photometry (AMDISEN 1967).

Results

In the table each value represents the average of duplicate determinations on 7 animals, the results are expressed in pmoles of cyclic AMP formed per mg protein in 15 minutes. The differences between the vasopressin-stimulated activities and the simultaneously determined unstimulated activities are presented. It appears that in group 1 and 3 the adenyl cyclase was stimulated to the same degree in the lithium treated and in the control rats. In group 2 there was less stimulation in the lithium treated than in the control rats (about 30 per cent less). The use of an ATP regenerating system led to an increased formation of cyclic AMP in both polyuric and control rats, but the difference between the groups remained unaltered.

At the time the analyses were carried out, the serum and kidney lithium levels were the same in groups 1 and 2, 0.6–0.8 mM in the serum and about 1 mM per kg wet weight in the kidney tissue. In group 3 the lithium levels had fallen to values not detectable with the methods used. It should further

Table 1

Renal ad l cyclase activity i particul'ing protei after 15 min.

Three groups of rats were treated with lithium before measurement of renal adenyl cyclase activities and compared with those of the control rats. Group 1 was treated for 7 days, and group 2 for 3 weeks until a pronounced polyuria had developed. In group 3 lithium treatment was stopped after 3 weeks and the analyses carried out a week later. The S.E.M. in the first two columns includes biological and day to day analytical variations while in the last column the S.E.M. is the variation of the mean of the seven differences.

		No	Unstimulated Mean	S.E.M.	Vasopressin Stimulation Mean	S.E.M.	Difference Mean	S.E.M.	Significance
Group 1	Control	7	130	1.7	221	14.1	91	9.0	NS
	Lithium	7	139	4.6	234	9.4	96	7.5	
Group 2	Control	7	124	5.5	216	9.1	92	7.3	P < 0.01
	Lithium	7	[illegible]	6.9	[illegible]	7.5	64	5.5	
Group 2R	Control	7	140	7.8	[illegible]	14.0	184	7.6	P < 0.01
	Lithium	7	155	5.9	[illegible]	14.1	136	11.9	
Group 3	Control	7	[illegible]	5.4	180	13.1	[illegible]	9.6	NS
	Lithium	7	105	7.0	[illegible]	8.9	67	5.0	

S.E.M. = Standard error of the mean.
R = ATP regeneration.
NS = Not significant

be noted that due to the washing procedure used in the preparation of the particulate fraction for enzyme analyses, no lithium could be detected even in the incubates from groups 1 and 2.

Discussion

In our previous paper (GEISLER *et al.* 1972) we discussed some of the factors which might be responsible for the decreased formation of cyclic AMP in incubates with tissue from polyuric rats. Lack of ATP *due to* other ATP requiring processes was among the possibilities mentioned. In the experiments presented here the difference between the polyuric and the control rats persisted in the presence of an ATP regeneration system.

In the present as in the previous experiments the difference between polyuric rats and control rats could be demonstrated with incubates where the lithium concentration was too low to be determined. This indicates that the

phenomenon studied does not depend on the presence of the lithium ion it self The serum and kidney lithium concentrations were the same in both groups of rats nevertheless there was no polyuria and no inhibition of the vasopressin-stimulated adenyl cyclase in group 1 whereas these phenomena were present in group 2

The results obtained with group 3 shows that both the polyuria and the lowered vasopressin-response of the adenyl cyclase are fully reversible.

Our observations indicate, that the lithium-induced vasopressin-resistant polyuria is caused, at least partly by the lithium-induced lowered response of kidney adenyl cyclase to vasopressin.

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We are grateful to Mrs. B Asmussen and Mrs. N Balslev for skilful technical assistance in the practical work.

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From the Departments of Histology, Pharmacology and Psychiatry II, University of Lund, Sweden

Effect of Chlorpromazine and Desmethylimipramine on the Synthesis of Noradrenaline from Dopamine in the Rat Heart

By

Lars Nordgren

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Key-word: Chlorpromazine – desmethylimipramine – noradrenaline – dopamine.

Following the administration of adrenaline and noradrenaline (NA), there is an accumulation of these amines in the peripheral sympathetically innervated tissues (RAAB & GIGEE 1955; EULER 1956; WHITBY *et al.* 1961; ANDÉN 1964) and the rate of uptake seems to be related to the blood flow as well as to the sympathetic nerve density (KOPIN *et al.* 1964). Catecholamine uptake is significantly reduced by pretreatment with chlorpromazine (CPZ) or desmethylimipramine (DMI), the latter being the most potent in this respect (e.g. CARLSSON & WALDECK 1965). The aim of this study was to investigate the effect of CPZ and DMI on dopamine (DA) uptake in the rat heart shortly after the DA administration as well as the effect of these drugs on the synthesis of NA from exogenous DA.

Adult Sprague-Dawley rats of either sex were used. The animals were kept in cages at 30–32° during the experiments. Animals receiving saline injections served as controls. CPZ HCl (25 mg/kg) or DMI HCl (25 mg/kg) was injected intraperitoneally 2 hours before DA HCl (2 mg/kg) intravenously. Some animals received DA only. The animals were killed by decapitation 10 min after the DA injections. The heart was then cut up and dried between filter paper to remove blood before homogenization. NA and DA were determined according to BERTLER *et al.* (1958) as modified by HÄGGENDAL (1963). Tissue specimens corresponding to those used for the chemical analyses were investigated by the histochemical method of FALCK & HILLARP (see FALCK & OWMAN 1965).

Dopamine (DA) and noradrenaline (NA) (μg/g wet weight ± SD) in the rat heart. Chlorpromazine (CPZ) or desmethylimipramine (DMI) was injected

Acknowledgements

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From the Department of Pharmacology AB Bofors Nobel-Pharma, Mölndal, and the Department of Pharmacology School of Medicine, Linköping, Sweden

Studies on Mechanical Actions of Mepivacaine (Carbocaine®) and its Optically Active Isomers on Isolated Smooth Muscle. Role of Ca^{++} and Cyclic AMP

By

G. Åberg and R. Andersson

(Received February 10, 1972; Accepted March 3, 1972)

Abstract. The effects of the local anaesthetic mepivacaine and its optically active isomers on mechanical activity as well as the uptake and mobilization of Ca^{++} on phosphorylase and phosphodiesterase activity and on the content of cyclic AMP have been studied in smooth muscle. In low concentrations L(+)-mepivacaine had a contracting action on the rat portal vein and the guinea pig taenia coli, whereas D(−)-mepivacaine had a predominantly relaxing action. The contracting action was probably associated with a mobilization of tissue bound Ca^{++} In higher concentrations both isomers had a marked relaxing action, the D(−)-isomer being the most potent. The relaxation was competitively counteracted by Ca^{++} Ba^{++} and Sr^{++} but not by K^{+} histamine or noradrenaline. The uptake of $^{45}Ca^{++}$ was reduced, D(−)-mepivacaine tended to be more potent than L(+)-mepivacaine. The content of cyclic AMP was increased by DL-mepivacaine in taenia coli and by L(+)-mepivacaine, but not by D(−)-mepivacaine in the portal vein. During isometric conditions of taenia coli the increased content of cyclic AMP was partly caused by an adrenergic β-receptor stimulation and partly by a Ca^{++}-depending reduction of the phosphodiesterase activity

Key words. Local anaesthetics – optical isomers – smooth muscle – Ca^{++} – cyclic AMP

In the isolated rat portal vein the local anaesthetic agent mepivacaine had a biphasic action on the myogenic activity (ÅBERG & WAHLSTRÖM 1972). The contractile action was not always associated with an increased spike activity of the cell membrane. Subsequently higher concentrations of the drug relaxed the muscle despite a persisting and increased electrical activity The L(+)-isomer had a stronger contractile effect than the D(−)-isomer The D(−)-isomer on the other hand had a more pronounced relaxing action. In this investigation our aim has been to study the mechanisms of the myogenic actions of mepivacaine and its isomers.

It is generally believed that contraction and relaxation of various kinds of muscles depend on changes in the concentration of free Ca^{++} in the myoplasm (SANDOW 1965, EBASHI & ENDO 1968). It has moreover been suggested that in the smooth muscle there is a competition between binding sites for Ca and local anaesthetic agents (WEISS *et al.* 1961, FEINSTEIN 1966).

There is also a relationship between Ca^{++} and cyclic AMP in smooth muscle as cyclic AMP stimulates a calcium binding mechanism: this action may explain the relaxing effect of adrenergic β-receptor stimulating agents on smooth muscle (ANDERSSON & NILSSON 1972).

We have investigated the relationship between the myogenic action of the mepivacaine isomers, Ca^{++} and cyclic AMP in smooth muscle. Our first aim was to investigate these relationships in vascular smooth muscle (portal vein of the rat). As the amount of smooth muscle tissue was a limiting factor in the determination of some metabolic parameters we also included studies on the guinea pig taenia coli.

Methods

Preparations of the portal vein were obtained from male Sprague Da ley rats weighing 180–210 g, and taenia coli preparations were taken from the guinea-pig caecum. Only female guinea pigs weighing 200–300 g were used. All the animals were killed by stunning and bleeding.

The "normal" Krebs solution contained (mM): Na 137.47, Ca^{++} 2.49, K 5.94, Mg^{++} 1.19, Cl 134.11, HCO_3 15.48, H_2PO_4 1.19 and glucose 11.5. The "K-high" Krebs solution contained 123 mM K and was obtained by replacing NaCl of the normal solutions with KCl. In the experiments in which the antagonism between calcium ions and local anaesthetics were studied on the guinea-pig taenia coli, a modified and depolarizing "KNO_3-Ringer" was used. In this solution there was no precipitation of calcium salts when the external Ca^{++}-concentration was increased (FERRARI & CARPENEDO 1968). The composition of the "KNO_3-Ringer" was (mM): K 157.6, Mg^{++} 2.0, NO_3 148.4, Cl 10.1, HCO_3 3.6, and glucose 5.6. The solutions were kept at 37° and were aerated with a gas mixture containing 97 % O_2 and 3 % CO_2. pH was 7.4.

Most experiments were performed with the muscles mounted in a muscle bath with a volume of 40 ml. The muscle were connected to isometric tension transducers (Grass FTO3B) and the mechanical activities of the preparations were recorded on a Grass polygraph. When the cyclic AMP content was determined in the rat portal vein the preparations were suspended in buffer solutions without being attached to any recording device.

$^{45}Ca^{++}$-uptake in taenia coli.
Pieces of taenia coli were incubated in ^{45}Ca-labelled Krebs buffer for different times. The muscles were then rapidly dipped into three successive beakers containing non-radioactive solutions similar to the incubation medium; they were then blotted on filter paper. The tissues were dissolved in 1 ml soluene and 9 ml of scintillation fluid was added (toluene containing 5 g PPO and 0.3 g dimethyl-POPOP per litre). The tissue

content of isotope was counted in a Packard Tri-Carb liquid scintillation spectrometer. The distribution of ^{45}Ca in the whole tissue is expressed by the following equation:

$$\text{distribution } (\%) = \frac{\text{content in wet tissue (dpm/mg)}}{\text{medium concentration (dpm/}\mu\text{l)}} \times 100$$

Myogenic action of drugs on the rat portal vein.

The muscle showed rhythmic contractions and relaxations. In order to measure the different degree of muscular activity quantitatively the product of tension (N) and time (min.) was calculated for 1 min. periods at certain pre-fixed times before and after the addition of local anaesthetics. The product was measured as the area under the tension curve with a planimeter and was expressed in per cent of the area that had been obtained during 1 min. when noradrenaline 1×10^{-6}g/ml had been added to the bath fluid. The spontaneous activity of the portal veins was also expressed in per cent of the maximal activity produced by noradrenaline.

Effects of local anaesthetics on cumulative dose-response curves for Ca^{++}

In the cumulative dose-response experiments on K -depolarized taeniae, increasing doses of $CaCl_2$ were added to the bath solution t 3 min. intervals until there was an auto-inhibitory effect of $CaCl_2$. The local anaesthetic was added to the bath 3 min. before the addition of the first dose of $CaCl_2$. In some experiments the adrenergic β-receptor blocking agent sotalol (4-(2-isopropylamino-1-hydroxyethyl)-methanesulfonanilide hydrochloride; LISH *et al.*, 1965) was used. This β-blocker has no local anaesthetic effects of its own (AMBLO & WELIM, 1967). It was added to the bath solution 6 min. before the first dose of $CaCl_2$.

Cyclic 3'5' AMP was analyzed according to the method of KAKIUCHI & RALL (1968), and phosphodiesterase ctivity according to KUKOWETZ & PÖCH (1970) but without correction for the dilution of the homogenate. The phosphorylase activity was determined according to BUEDING *et al.* (1962).

The local anaesthetics used in this investigation were DL-mepivacaine (M = 283) L(+)-mepivacaine, D(−)-mepivacaine.

Results

Rat portal vein

Myogenic action of the mepivacaine isomers

In fig. 1 a comparison is shown between the products of tension x time (for explanation see "Methods") for the L(+)- and D(−)-isomers of mepivacaine. In the lower concentration (3×10^{-4} g/ml) L(+)-mepivacaine had a predominantly contracting action on the muscle, whereas the D(−)-isomer had a relaxing action. In the higher concentration (3×10^{-3} g/ml) both isomers produced a marked relaxation.

Influence of Ca^{++} on the relaxing action of mepivacaine.

After treatment with mepivacaine (3×10^{-4} g/ml for 10 min.) the did not respond to other contracting agents such as noradrenaline.

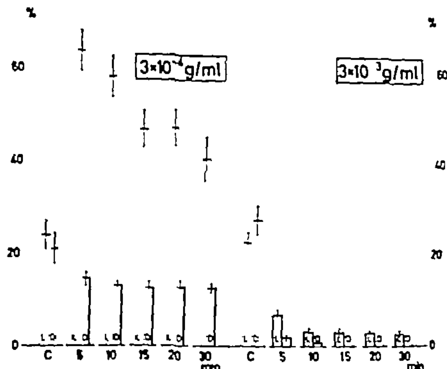


Fig. 1. Myogenic effect of L(+)- and D(−)-mepivacaine on the rat portal vein, expressed in per cent of the maximal myogenic effect of the individual muscles during noradrenaline (1×10^{-3} g/ml)-induced contractions.
C = Spontaneous myogenic activity.
Bars marked with 5, 10, 15, 20, 30 show the myogenic activity (means ± S.E.M.) at different times after addition of L(+)- or D(−)-mepivacaine to the bath fluid.

or K^{+} (fig. 2). Moreover when the portal vein was relaxed by mepivacaine, the addition of Ca^{++} to the bath produced a contraction. Ca^{++} could be substituted for Ba^{++} or Sr^{++} (fig. 2). The relaxing action of mepivacaine was thus specifically counteracted by calcium ions or by ions which have been shown *to substitute for calcium ions in the contractile process* (EBASHI & ENDO, 1968).

Relaxing action of the mepivacaine isomers on the potassium contracted portal vein

If the muscle was contracted by K^{+} (128 mM) the contracting action of the mepivacaine isomers was absent, though the relaxing effect was still present. There was a significant difference between the relaxing actions of the two isomers, the D(−)-form being 2–3 times more potent than the L(+)-form (fig. 3).

Influence of adrenergic β-receptor blockade on the relaxing action of the mepivacaine isomers

To test whether an adrenergic β-receptor stimulation was involved in the

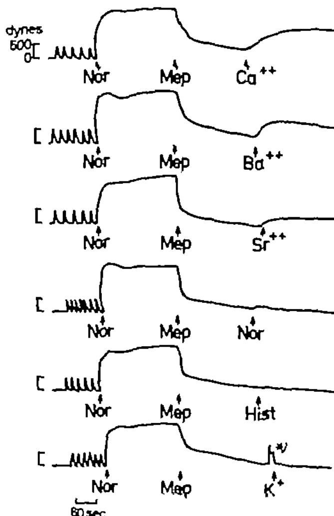


Fig. 2. Antagonism between the relaxing action of mepivacaine (Mep) 5×10^{-4} g/ml and contracting agents in the rat portal vein.

Nor = noradrenaline 1×10^{-6} g/ml.

Hist = histamine 1×10^{-4} g/ml.

The concentration of Ca^{++} Ba^{++} and Sr^{++} was 8 mM, the concentration of K was 128 mM

*) Artefact due to change of solution.

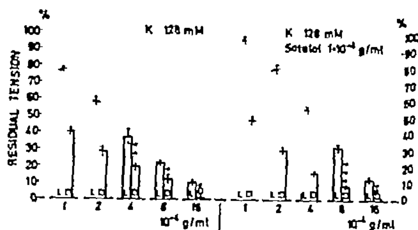


Fig. 3. Inhibitory effect of the mepivacaine isomers in increasing doses on K-contracted rat portal vein. Tests without and with pretreatment with sotalol. The bars show mean ± S. E. M. from 8 experiments. Initial tension in K-contracted muscle = 100 %. The significance of the difference between the isomers is shown in the figure, where *** means $P < 0.001$ means $P < 0.01$ and means $P < 0.05$.

relaxing action of mepivacaine, the K-contracted portal vein was treated with sotalol. Sotalol significantly diminished the relaxing action of L(+)-mepivacaine but not that of D(−)-mepivacaine (fig. 3).

Influence of the mepivacaine isomers on the content of cyclic AMP in the portal vein.

Since adrenergic β-receptor blockade reduced the relaxing action of the L(+)-isomer but not of the D(−)-isomer it was of interest to determine the effect of the two isomers on the content of cyclic AMP of the portal vein. After incubation of the preparation for 10 min., L(+)-mepivacaine (3×10^{-4} g/ml) increased the content of cyclic AMP by $0.75 \pm 0.30 \times 10^{-9}$ mol/g wet weight from a basal value of $1.80 \pm 0.20 \times 10^{-9}$ mol/g wet weight ($n = 8$, $P < 0.05$). The D(−)-isomer was ineffective: the change in cyclic AMP content was $+0.1 \pm 0.5 \times 10^{-9}$ mol/g wet weight. If the portal veins were pretreated with sotalol (1.2×10^{-5} g/ml) the increasing action of the cyclic AMP content was completely blocked. The change in cyclic AMP after the addition of the L(+)-isomer was $-0.6 \pm 0.4 \times 10^{-9}$ mol/g wet weight in the presence of sotalol. There was a statistically significant difference ($P < 0.02$) in the content of cyclic AMP between muscles treated with L(+)-mepivacaine (without sotalol) and those pretreated with sotalol.

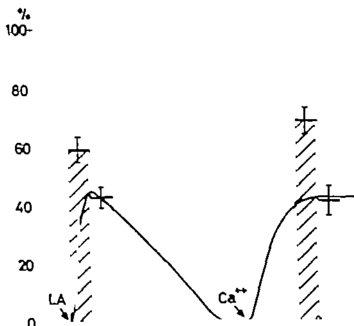


Fig. 4. Maximal contractile force elicited by the mepivacaine isomers (1×10^{-8} g/ml) in the isolated guinea pig taenia coli (left bars). Tension is expressed in per cent of the maximal contractile force by carbachol (1×10^{-7} g/ml) in the individual muscles.
Striped bars = experiments with L(+)-mepivacaine (= 11); open bars = experiments with D(−)-mepivacaine (n = 11).
15 min. after the addition of the local anaesthetic agent to the bath fluid, Ca^{++} (4 mM) was added. The maximal contractions after the administration of Ca^{++} were also expressed as per cent of the carbachol-contractions and are shown in the figure (right bars).
The curved line shows the finding from typical experiment with D(−)-mepivacaine (= LA) and Ca^{++}

Guinea pig taenia coli

Myogenic action of L(+)- and D(−)-mepivacaine.

The initial contracting action of the mepivacaine isomers was stronger in the taenia coli than in the portal vein. Taenia coli, but not the portal vein, was contracted by both isomers in a concentration of 1×10^{-8} g/ml. There was a significant ($P < 0.01$) difference between the maximal contractile effects of the isomers in this concentration on taenia coli, the L(+)-isomer was most potent (fig. 4)

After a short time (5–10 min.) the muscles relaxed, but the relaxation was antagonized by Ca^{++} The contracting effect of Ca^{++} was less inhibited by the L(+)-isomer than by the D(−)-isomer: $P < 0.001$ (fig. 4).

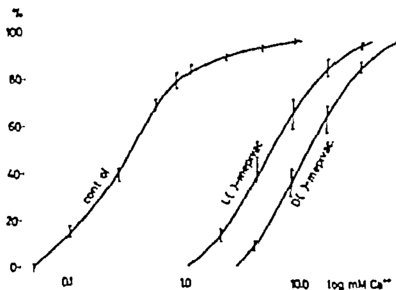


Fig. 5. Effects of mepivacaine isomers (1×10^{-5} g/ml) on the logarithmic concentration response curve for Ca in K-depolarized guinea pig taenia coli. Each point represents mean ± S. E. M. of 9–11 experiments.

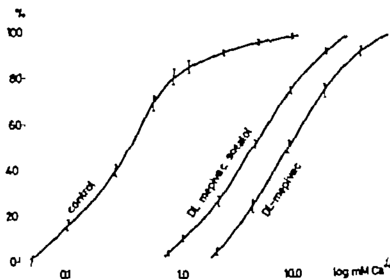


Fig. 6. Action of sotalol (5×10^{-6} g/ml) on the inhibitory effect of DL-mepivacaine (1×10^{-6} g/ml) on the logarithmic concentration-response curve for Ca^{++} K-depolarized guinea pig taenia coli. Each point represents mean ± S. E. M. of 9–12 experiments.

Influence of Ca^{++} on the myogenic effect of mepivacaine.

As shown in fig. 4 there was an antagonism between the actions of mepivacaine and Ca^{++} in taenia coli as well as in the portal vein (fig. 2) This could be studied in a more quantitative manner in the taenia coli than in the portal vein. In these experiments the muscle was made calcium-poor by incubation for 10 min. in a calcium-free and depolarizing (157 6 mM K) buffer solution. On addition of Ca^{++} in increasing concentrations, the muscle contracted according to the "control curve of fig. 5 Pretreatment of the muscle with mepivacaine shifted the dose response curve of calcium to the right, but it was still possible to obtain the maximal contractile effect of calcium. This change of the dose-response curve might indicate a competitive antagonism. The D(−)-isomer was about 3 times as potent as the L(+)-isomer in antagonizing Ca^{++} (fig. 5).

To investigate the possible role of adrenergic β-receptor stimulation in the relaxing effect of mepivacaine, some of the muscles were pretreated with sotalol (5×10^{-6} g/ml) The dose-response curve for calcium in the presence of mepivacaine was then shifted to the left (fig. 6). There was no effect of sotalol on the control curve. Adrenergic β-receptor blockade thus reduced the mepivacaine-induced inhibition of the calcium-contraction.

$^{45}Ca^{++}$-uptake in taenia coli

If the tension of the muscle is a function of the myoplasmic concentration of free" Ca^{++} the results presented in fig. 5 and 6 indicate that mepivacaine in some way reduced the increase in myoplasmic Ca^{++} on addition of external Ca^{++} The uptake of $^{45}Ca^{++}$ from normal Krebs solution was therefore studied The uptake was significantly reduced by D(−)-mepivacaine after 5

Table 1

Effects of L(+)- and D(−)-mepivacaine (3×10^{-4} g/ml) on $^{45}Ca^{++}$-uptake in taeniae coli. The control values show the distribution of ^{45}Ca in per cent (for explanation see "Methods").

The significance of the changes from the control values are shown in the table, where ** means $P < 0.01$ and means $P < 0\ 05$.

Time of incubation with ^{45}Ca (min)	N	Control value	Change from control value elicited by L(+)-mepivacaine	Change from control value elicited by D(−)-mepivacaine
5	9	89 1 ± 5.0	− 12.4 ± 6.5	− 16.7 ± 4.2**
15	7	157.3 ± 8.0	− 15.2 ± 5.5	− 22.9 ± 7 4
30	8	167.6 ± 23.7	+ 1.9 ± 10.7	− 21.8 ± 10.1
60	9	171.4 ± 10.0	− 9.0 ± 10.2	+ 0 9 ± 8.4*

min. ($P < 0.01$) and 15 min. ($P < 0.05$) and by L(+)-mepivacaine after 15 min ($P < 0.05$). There was no significant reduction in the uptake of $^{45}Ca^{++}$ after incubation for 30 min. or 60 min. (table 1)

Effect of mepivacaine on Ca⁺⁺ release

HURWITZ *et al.* (1967) demonstrated in intestinal smooth muscle that if the muscle was exposed to Ca⁺⁺ at a high external concentration (36 mM) it was initially contracted but then relaxed. If the external Ca⁺⁺-concentration thereafter was reduced to zero the muscle contracted again. The contraction in the Ca⁺⁺-free buffer solution was attributed to a mobilization of tissue bound Ca⁺⁺.

The corresponding phenomena were found in our experiments on taenia coli. The contraction elicited by changing to the Ca⁺⁺-free buffer solution was increased by 166 ± 39 per cent ($P < 0.001$, $n = 8$) if the buffer contained DL mepivacaine 3×10^{-4} g/ml (fig. 7). This experiment indicated that the contractile action of mepivacaine was associated with a mobilization of bound Ca⁺⁺.

Metabolic actions of mepivacaine in taenia coli

The influence of sotalol on the mepivacaine induced inhibition of Ca⁺⁺ contractions (fig. 6) indicated that the cyclic AMP system was involved in the relaxing action of mepivacaine.

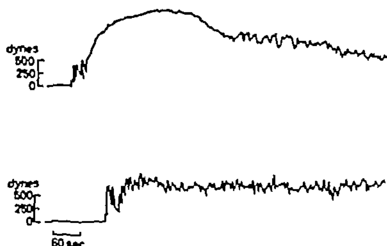


Fig. 7. Taenia coli preparations contracting in Ca⁺⁺-free solution after preincubation for 60 min. in Ca⁺⁺-high (40 mM) solution.
Upper curve: Tension developed in Ca⁺⁺-free solution containing DL mepivacaine 3×10^{-4} g/ml.
Lower curve: Control experiment in Ca⁺⁺-free solution without mepivacaine, using another preparation of taenia coli from the same animal.

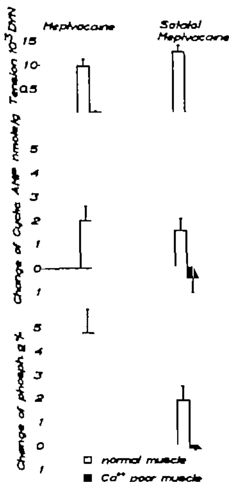


Fig. 8. Influence of mepivacaine (3×10^{-4} g/ml) alone and in combination with sotalol (1.2×10^{-5} g/ml) on tension, cyclic AMP content and phosphorylase activity in normal and Ca^{++}-poor guinea-pig taeniae coli 90 sec. after addition of the drug. Mean ± S. E. M. (n = 5–8).

After incubation with DL-mepivacaine (3×10^{-4} g/ml) for 90 sec. the content of cyclic AMP in isometrically contracted taeniae coli was increased by 2.04 ± 0 72 ($P < 0.05$) from a mean basal value of 5 49 ± 0.66 (fig. 8). The increase in cyclic AMP produced metabolic effects in the muscle since it was found that the phosphorylase *a* activity was also increased (fig. 8). If the adrenergic β-receptors were inhibited by sotalol however the actions of mepivacaine were not blocked (fig. 8)

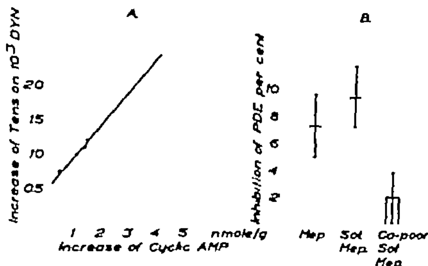


Fig. 9 A. Relationship between the increase in cyclic AMP content and the increase of tension in taeniae coli, pretreated with sotalol (1 × 10⁻⁵ g/ml). The analysis are performed 90 sec. after addition of mepivacaine (3×10^{-4} g/ml).
B. Inhibitory effect of mepivacaine (3×10^{-4} g/ml) on phosphodiesterase activity in normal and Ca -poor taeniae coli. Tests without and with pretreatment with sotalol (1 × 10⁻⁵ g/ml). Mean ± S.E.M. (n = 5–7).

There was a correlation between the increased tension and the increased content of cyclic AMP in the presence of mepivacaine (fig. 9A). The drug also reduced the phosphodiesterase activity (fig. 9B). In order to investigate if this reduction was caused by the high concentration of "free" myoplasmic Ca to which the contractile effect of mepivacaine was related, experiments on Ca -poor preparations were performed since contracting agents do not increase the content of cyclic AMP in such preparations (ANDERSSON unpublished results). Mepivacaine, however, still increased the cyclic AMP content and the phosphorylase *a* activity although its effect on the tension was blocked (fig. 8). However, if sotalol was added to the Ca -poor muscle the metabolic effects of mepivacaine were blocked (fig. 8). The increasing effect of mepivacaine on cyclic AMP and phosphorylase *a* in isometrically contracting taenia coli was thus dependent both on the stimulation of adrenergic β-receptors and on an inhibition of the phosphodiesterase activity that was elicited by the increased concentration of myoplasmic Ca^{++}.

Discussion

The mechanism of action of agents with nerve blocking properties has been widely discussed. In a recent review by RITCHIE (1971) alternative

hypotheses have been thoroughly presented and the role of Ca^{++} discussed. No single hypothesis could explain all the observed facts.

In earlier experiments we found (ABERG & WAHLSTRÖM 1972) that there was no initial inhibition of the electrical spike activity by local anaesthetics in smooth muscle membranes. Instead this activity was increased whether the muscle was contracted or relaxed The contractile action was probably related to the increase in the electrical activity The relaxing effect was not due to an inhibition of the spike activity of the smooth muscle membrane, as the relaxing effect was produced before any inhibition of the electrical activity of the muscle membrane was evident (ABERG & WAHLSTRÖM 1972) Further more we have found that there was no influence of mepivacaine on the contracting effect of ATP on glycerinated taenia coli preparations (ABERG, to be published). These facts indicate that the relaxing action of the local anaesthetic agents in smooth muscle was independent of the electrical activity of the smooth muscle membrane and also of the effects on the contractile proteins, but probable influenced the excitation-contraction coupling mechanism.

If contraction and relaxation in smooth muscle is regulated by the concentration of free myoplasmic Ca^{++} then the interaction between local anaesthetic agents and Ca^{++} is of primary interest. FEINSTEIN (1963 & 1966) has reported that procaine and tetracaine reduced the efflux of ^{45}Ca from frog sartorius muscle and rat uterus. Our studies indicate that both isomers of mepivacaine reduced the uptake of $^{45}Ca^{++}$ in taenia coli and also competitively inhibited the contractile effect of Ca^{++} in K -depolarized muscle (fig. 5 & 6). These results indicate that the passage of Ca^{++} through the cell membrane was reduced by mepivacaine the D(−)-isomer tended to be more potent than the L(+)-isomer FEINSTEIN & PAIMRE (1969) and THORPE & SEEMAN (1971) have reported that local anaesthetics inhibit the binding of Ca^{++} to the sarcolemma in striated muscle. This indicates a competition for carrier sites in the cell membrane of muscle tissue.

An inhibitory action of mepivacaine on the Ca^{++}-passage through the cell membrane cannot, however explain all the observed effects of the compound on smooth muscle. The results presented in fig. 7 for example indicate a mobilization of cellular Ca^{++} and in low concentrations mepivacaine had a contracting effect despite the fact that the inflow of external $^{45}Ca^{++}$ was significantly reduced. JOHNSON & INESI (1969) demonstrated that tetracaine in a high concentration increased the efflux and reduced the net Ca^{++} accumulation of the sarcoplasmic reticulum of rabbit skeletal muscle and THORPE & SEEMAN (1971) observed that procaine displaced Ca^{++} passively bound to the reticulum. If local anaesthetics also have these effects on intracellular Ca^{++}-stores, an inhibition of the Ca^{++}-passage through intracellular membranes must be assumed too.

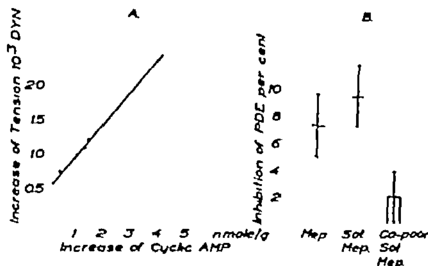


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B. Inhibitory effect of mepivacaine (3×10^{-4} g/ml) on phosphodiesterase activity in normal and Ca -poor taeniae coli. Tests without and with pretreatment with sotalol (1.2×10^{-4} g/ml). Mean ± S.E.M. (n = 5–7).

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Acknowledgement

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From the Department of Food Hygiene and the Department of Anatomy and Embryology College of Veterinary Medicine, Helsinki, and the Electron Microscope Laboratory University f Helsinki, Finland

Ultrastructure and Microsomal Protein Content of Mouse Liver Treated with Methyl Mercury*

By

Timo J.Pekkanen and Lars-Axel Lindberg

(Received June 16, 1972; Accepted June 23, 1972)

Abstract 24 male NMRI mice weighing about 17 g each were divided into two equal groups. Group 1 was treated intraperitoneally with methyl mercury nitrate. The dose was 0.3 mg Hg/kg in 0.5 ml water Group 2, which was kept as a control, was treated with 0.5 ml water intraperitoneally After 7 days all the mice were sacrificed. As seen by electron microscopy the smooth endoplasmic reticulum was proliferated and vesicular in all the mice treated with methyl mercury The mean liver microsomal protein content was significantly increased in group 1 as compared to group 2 (the controls).

Key-words: Methyl mercury – microsomes – endoplasmic reticulum.

Previous work showed that a single administration of methyl mercury significantly decreased the duration of hexobarbital (enhexymalum NFN) hypnosis in rats when hexobarbital was administered one week after the methyl mercury treatment (PEKKANEN & PEKKARINEN 1972). There are several drugs, known as inducers of liver microsomal drug metabolism, which produce this effect (CONNEY 1967)

Chronic administration of these inducers to experimental animals leads to an increase in the microsomal fraction of the liver and to hypertrophy of the smooth endoplasmic reticulum (SER) which is part of the microsomal fraction. The microsomal protein content of the liver increases, as does the activity of several drug-metabolizing enzymes of the liver microsomes (CONNEY 1967 CONNEY *et al.* 1960)

The purpose of this investigation was to study the effect of a single methyl mercury treatment on the ultrastructure of the liver and on the microsomal protein content of the liver of mice.

This work was partly supported by the Emil Aaltonen Foundation, Tampere, Finland.

Material and methods

Male NMRI-mice (24) weighing about 17 g (range 14.1–20.1 g) were divided into two equal groups. Group 1 received methyl mercury nitrate, in a dose of 0.3 mg Hg/kg, intraperitoneally in about 0.5 ml water and group 2, the control group was given 0.5 ml water also intraperitoneally. Before and during the experiment the mice were fed a standard laboratory diet *ad libitum* (Orion, Helsinki, Finland). After 7 days all the mice were sacrificed under light ether anaesthesia by severing the abdominal aorta.

For examination of ultrastructure small cubes of liver were fixed in 3 % glutaraldehyde buffered with 0.1 M phosphate buffer for 2 hours at 4° and pH 7.2. The samples were then rinsed in phosphate-buffered 0.2 M sucrose, pH 7.2, for 24 hours

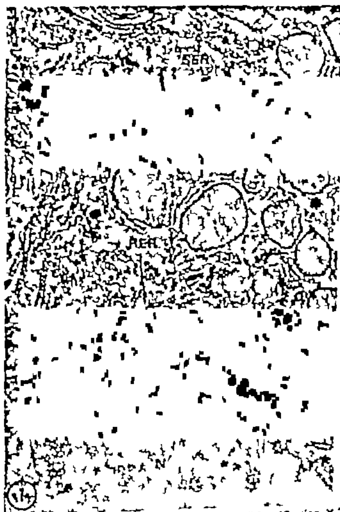


Fig. 1. Electron micrograph of liver from control mouse (Magnification × 22,000). N, Nucleus; M, mitochondrion; RER, rough endoplasmic reticulum, SER, smooth endoplasmic reticulum.

at 4°. After dehydration with ethyl alcohol the samples were embedded in Epon (LUFT 1961). Sections cut on a LKB ultrotome were stained with lead citrate (REYNOLDS 1963). The sections were examined in an Akashi Tronscope and a Philips EM 200 electron microscope (fig. 1 & 2). From the remainder of each liver a 10 % (w/v) suspension was prepared in an ice-cold homogenizing medium consisting of 0.25 M sucrose in 0.05 M phosphate buffer pH 7.4. The homogenization was performed with a Thomas tissue grinder (A. H. Thomas, Philadelphia, USA) for 30 seconds and with 10 up and down movements with the pestle. One ml aliquot of this homogenate was used for the total protein determination (LOWRY *et al.* 1951). Microsomes of the homogenate were further prepared as described by NELSON *et al.* (1971). The microsomal protein was determined according to LOWRY *et al.* (1951).



Fig. 2. Electron micrograph of liver from mouse 7 days after single treatment methyl mercury (Magnification × 22,000). SER seems proliferated, RER is

Results

As seen by electron microscopy the amount of the smooth endoplasmic reticulum seemed to be increased and it became vesicular in nature in all the mice treated with methyl mercury (fig. 2) Parallel arrays of rough endoplasmic reticulum had to a great extent disappeared.

The mean liver microsomal protein content was 83.20 ± 9.96 mg/g (mean $\pm$ S) liver protein in group 1 treated with methyl mercury and 68.37 ± 8.30 mg/g in the control group. The difference between the means is significant ($P < 0.01$ Student's t test).

Discussion

The administration of methyl mercury to mice led to hypertrophy of the smooth endoplasmic reticulum in the hepatocytes and to an increase in the amount of microsomal protein. This is a common property of drugs known as inducers of microsomal metabolism (CONNEY 1967) The results obtained thus give further evidence of the character of methyl mercury as an inducer of liver microsomal drug metabolism.

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From the Department of Pharmacology Royal Veterinary College,
S-104 05 Stockholm 50, Sweden

Age Dependent Changes in Nicotine Distribution in the Brain of the Mouse

By

Torbjörn Ståhlhandske and Premysl Slanina

(Received November 19 1971 Accepted December 22, 1971)

Abstract: Mice of different ages, from newborn to 26-day old, were injected intraperitoneally with 1 mg/kg of ^{14}C-nicotine and the nicotine concentrations in the blood and brains were determined 10 minutes after the injection. In 3-day old and 12-day old mice the nicotine concentrations in the blood and brains were also determined 1, 2.5, 5 20 and 60 minutes after the injection. In addition, the nicotine distribution in 3-day old, 12-day old and 26-day old mice was studied by whole-body autoradiography. The results showed that the ability of the brain to accumulate nicotine increases with age. In addition there were also changes in the distribution of nicotine within the brain.

Key-words: Nicotine – brain – distribution – age factors.

In a previous study by STÅLHANDSKE *et al.* (1969), it was shown that the tolerance to the lethal effects of nicotine in mice decreased from 3 to 12 days of age despite a concomitant increasing capacity for nicotine detoxication. A possible explanation for this phenomenon was presented in a later study by STÅLHANDSKE & SLANINA (1970), in which lethal brain concentrations of nicotine in mice of various ages were determined. It was found that lethal nicotine concentration in the brain at 12 days of age was significantly lower than at 3 days of age, suggesting an age-dependent decrease in brain tolerance to the lethal effects of nicotine.

Apart from this change in brain tolerance, other results in the same study suggested the presence of an additional variable condition responsible for a decrease of the dose necessary to produce a lethal effect. It was found that brain to blood ratios at death varied with age in a way suggesting an increasing ability of the brain to concentrate nicotine with increasing age. The purpose of the present study was to analyze this phenomenon more closely

Methods

Compounds.

Nicotine N-methyl-14C was obtained from the Radiochemical Centre, Amersham, England. The specific activity of the substance was 20 mci/mM. The radiochemical purity determined by autoradiography of thin-layer chromatograms (STÅLHANDSKE 1970a) was found to be above 98 %.

Animal experiments.

Mice of the NMRI strain kept on a standard pelleted diet were used. Pregnant mice were bought from a local breeder. The litter size used in the experiments ranged from 6 to 9 young and the young were kept with their mothers until used. Isolated pregnant females were inspected in the morning and the animals were considered to be newborn (0 days) when they were first found. This implies that the actual age of the animal might be anywhere from 0 to 24 hours more than the age attributed to it in this work. Experimental animals of the estimated age of 0 (newborn) 1 3 5 7 9 1.. 17 and 26 days were used and no division of sexes was made. The CNS of .6-day old mice was considered to be mature and representath of the adult. This was considered relevant since KOBAYASHI et al. (1963) have demonstrated that by 17 days, several important parameters of neurological development reach maturity. In addition FOX (1965) stated that from 16 days of age the manipulative abilities are adult-like and that reflex responses are of the adult type at 17 to 18 days of age.

The animals were injected intraperitoneally with 14C-nicotine dissol ed in distilled water. The injected dose of nicotine base was 0.5 1.0 or 1.5 mg/kg and the volume 0.005 ml/g body weight. Animals of 0 to 12 days of age were injected with a Hamilton 100 μl microsyringe and the site of injection was pinched to avoid leakage in the youngest animals. The animals were sacrificed by cervical dislocation and immediately bled by decapitation. The blood was collected in 3 ml of heparinized ice cold 0.2 M phosphate buffer pH 7.4. In 1 and 3-day old animals blood from 3 animals was pooled to make one sample.

The animals were killed 1 2.5, 5, 10, 20, and 60 minutes after the injection. The brain and blood were removed weighed and immediately frozen and kept at −20 until the determination of nicotine. The brain was defined as the part of CNS above the foramen magnum.

For dry-weight determinations, 8 brains of 3-day old 5-day old, and 12-day old mice were kept in an oven at 90 and then cooled down in a vacuum desiccator. This procedure was repeated until a constant weight of the sample was obtained.

Determination of nicotine

The brains were homogenized in 0.2 M phosphate buffer pH 7.4. From the homogenates and blood samples 14C-nicotine was extracted according to the method of HUCKER et al. (1960). The amount of radioactive nicotine thus extracted was determined in a Packard Tri-Carb liquid scintillation counter after addition of Packard Instagel. The extraction was checked by thin-layer chromatography (STÅLHANDSKE 1970a).

Whole-body autoradiography

Three animals aged 3, 12, and 26 days were sacrificed 5 minutes after the intraperitoneal injection of 0.5 μci of 14C-nicotine/g body weight corresponding to 0.004 mg/g of nicotine base. After freezing the animals, they were sectioned and auto-

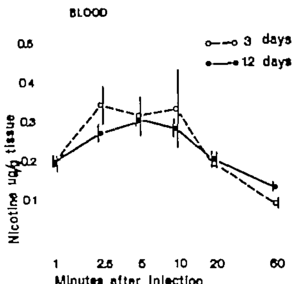


Fig. 1. Nicotine concentrations in the blood of 3-day old and 12-day old mice at various times after the intraperitoneal injection of ^{14}C-nicotine. The values in the figure represent the average of 8 in 12-day old and 9 in 3-day old animals. Vertical bars are standard errors.

radiographed according to the Ullberg method (ULLBERG 1954 & 1958). The exposure time varied from 4 to 5 weeks.

I adult animals, about 70 % of the total radioactivity in the brain (STÅLHANDSKE 1970b), 5 minutes after the intraperitoneal injection, represents nicotine. The remainder is due to the main nicotine metabolite cotinine, which has an even brain distribution quite different from nicotine (BOWMAN et l. 1964). In 12 and 3-day old mice th cotinine epresents less than 3 % of the total radioactivity in the brain (STÅLHANDSKE, unpublished results).

Statistical evaluations of data.

Differences between the means were tested with Student's t-test. The level of significance used was $P < 0.05$

Results

The nicotine concentrations in the blood after the intraperitoneal injection of 1 mg/kg nicotine did not differ significantly in 3- and 12-day old mice at any time of observation (fig. 1) However nicotine concentrations in brains of 12-day old mice were significantly higher than the nicotine centrations in the brains of 3-day old mice at 2.5 5 and 10

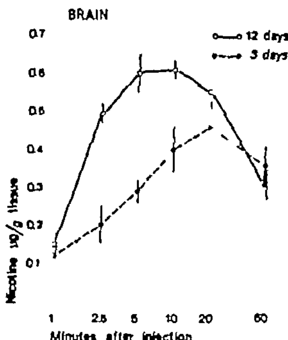


Fig. 2. Nicotine concentrations in the brains of 3-day old and 12-day old mice at various times after the intraperitoneal injection of ^{14}C-nicotine. The values in the figure represents the average of 8 animals. Vertical bars are standard errors.

injection ($P < 0.001$) (fig. 2). A maximal difference occurred 2.5 minutes after the injection, when the average nicotine concentration in the brains of 12-day old mice exceeded that of the 3-day old mice by two and a half times. It was also seen that the maximum nicotine concentration was lower and occurred later in the 3-day old animals. In both 3- and 12-day old mice the concentrations of nicotine in the brain increased linearly with the dose administered and the brain to blood ratios were constant with the exception of a small, but significant decrease for the 12-day old mice at the highest dose (fig. 3). However the brain-to-blood ratios of 12-day old mice were significantly higher at each dose level (table 1). On an average they exceeded those of 3-day old mice by a factor of 2.4.

The intraperitoneal injection of nicotine into mice of various ages revealed that nicotine concentrations in the blood 10 minutes after the injection did not change significantly with age from birth up to the age of 12 days (fig. 4). However at the ages of 17 and 26 days, significantly lower concentrations were observed. The nicotine concentrations in the brain increased slightly during the first 5 days after birth, but increased rapidly from that time up to the age of 12 days (fig. 4). In the brain of

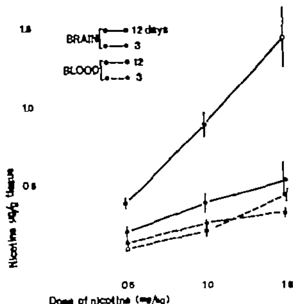


Fig. 3. Nicotine concentrations in the brains and blood of 3-day old and 12-day old mice 10 minutes after the intraperitoneal injection of various doses of ¹⁴C-nicotine. The values in the figure represent the average of 8 in 12-day old and 9 in 3-day old animals. Vertical bars are standard errors.

older mice (17 and 26 days) a decrease in the concentration of nicotine was observed. The brain-to-blood ratios steadily increased from 1.3 at birth up to 5.5 at 26 days. The most rapid increase was seen during the period between 5 and 12 days.

To investigate whether the rapid increase of nicotine in the brain was

Table 1

Average brain/blood ratios ± S.E.M. in 3 and 12-day old mice 10 minutes after the intraperitoneal injection of nicotine.

Age (days)	No. of animals	Dose mg/kg 0.5	1.0	1.5
3	9	I 1.56 ± 0.10	II 1.49 ± 0.06	III 1.67 ± 0.10
12	8	IV 3.92 ± 0.05	V 3.96 ± 0.04	VI 3.27 ± 0.17

I–IV II–V III–VI, V VI $P < 0.001$

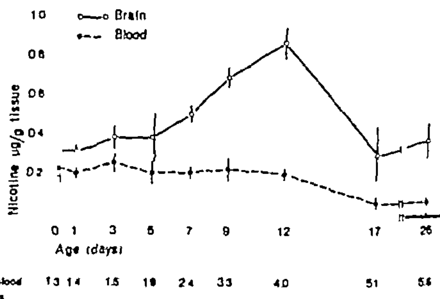


Fig. 4 Nicotine concentrations in the blood and brains of mice of various ages, 10 minutes after the intraperitoneal injection of ^{14}C-nicotine. The values in the figure represent the average of 9 to 8 animals. Vertical bars are standard deviations.

due to an increase in solid brain constituents, the water content of the brain was determined (table 2). The results showed that the change in the water content with change in age was not responsible for the increase observed. The nicotine content in the brain of 12-day old animals per

Table 2

Average water content and micrograms of nicotine per gram dry weight ± S.E.M. in the brains 10 minutes after intraperitoneal injection of nicotine.

Age (days)	No of animals	Water content (%)	Nicotine µg/g dry weight
3	8	87.9 ± 0.1	3.34 ± 0.17
5	8	87.0 ± 0.1	3.09 ± 0.36
			$P < 0.001$
12	8	83.9 ± 0.1	5.50 ± 0.18

gram dry weight as well as per gram wet weight, was significantly greater than that for 3-day old animals.

Autoradiography

Autoradiographic studies of the CNS of 3- 12 and 26-day old mice, 5 minutes after the intraperitoneal injection of ^{11}C-nicotine, are shown in figures 5a–c. In the 3-day old mouse (fig. 5a), high levels of radioactivity were observed in the medulla spinalis and medulla oblongata, while on the other hand, radioactivity was very low in the forebrain structures. High radioactive concentrations, not seen in the picture, were also seen in the hippocampal formation lobus olfactorius and cerebellum. Again in the 12-day old mouse (fig. 5b) the highest levels of radioactivity were seen in the medulla and lobus olfactorius, but in this case the radioactivity in the forebrain structures was comparatively higher than in the 3-day old mouse. A high radioactive concentration was also seen in the hippocampal formation. However in the CNS of the 26-day old mouse (fig. 5c) a quite different pattern of distribution was seen. Here the forebrain structures had the highest level of radioactivity and the medulla, the lowest. At this age too the radioactivity was high in the hippocampal formation.

Discussion

The present study reveals that the accumulation of nicotine in the mouse brain after intraperitoneal administration is significantly changed with a change in age. From birth up to 12 days of age there is a steady increase in the absolute brain concentrations as well as a higher accumulation of nicotine relative to the blood levels. After 12 days there is still an increasing accumulation of nicotine relative to the blood levels, but the absolute concentrations decrease. The decrease in absolute concentrations during this period is evidently due to a decrease in the blood levels, which is influenced by the hepatic metabolism of nicotine, which is rapidly increasing during this period of life (STÅLHANDSKE *et al.* 1969)

The study of nicotine accumulation in the brains of 3- and 12-day old mice at different times after the injection, shows that in the former the uptake is slower and the maximal concentrations are lower However the blood concentrations do not differ indicating similar conditions of absorption.

This age-dependent increase of nicotine accumulation in the brain is evidently a contributing factor to the concomitant decrease of tolerance to the lethal effects of nicotine as observed by STÅLHANDSKE *et al.* (1969) In this study it was shown that 12-day old animals had a lower tolerance

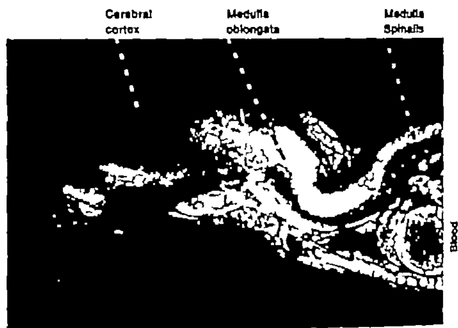


Fig. 5a

Cerebellum
Medulla oblongata
Medulla spinalis
Cerebral cortex
Blood

Fig. 5b

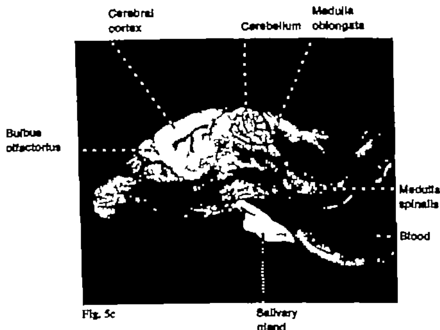


Fig. 5c

Fig. 5a–c. Autoradiograms showing the distribution of radioactivity in the CNS of 3-day old (a), 12-day old (b) and 26-day old mouse (c) 5 minutes after the intraperitoneal injection of ^{14}C-nicotine. Note the different pattern of distribution at the various ages.

to nicotine than either 3- or 24-day old animals, which is in accordance with the present findings, which similarly show the highest concentrations of nicotine in the brains of the 12-day old animals. In a study by STÅLHANDSKE & SLANINA (1970) it was further observed that after the administration of a lethal dose there was a much shorter latent period before death in 12-day old mice as compared with 3-day old mice. A contributing factor to this difference is very likely the comparatively slow accumulation of nicotine observed in the brains of 3-day old mice.

This change with age of components which govern total accumulation of nicotine in the brain has also been observed in similar studies with other drugs. FIREMARK *et al.* (1963) found that the total accumulation of diphenylhydantoin increases in the course of maturation. In addition they found an increased binding of diphenylhydantoin in the brain and blood with increasing age. A similar phenomenon, probably due to the neurochemical and structural maturation of the CNS, may also be of importance with regard to the distribution of nicotine. However with nicotine, an age-dependent change of binding in plasma is unlikely since preliminary studies have

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From the Oakdale Toxicology Center Department of Pharmacology
College of Medicine, University of Iowa, Iowa City Iowa 52240, U.S.A.

Infusion of Morphine in Maternal Rats at Near Term Maternal and Foetal Distribution and Effects on Analgesia, Brain DNA RNA and Protein

By

Torkell Jóhannesson, William J Steele and Bernard A. Becker
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Abstract Time-dated pregnant (day 21-22) Sprague-Dawley rats were infused intravenously with morphine 5 mg/kg per hr for periods up to 4 hrs. The blood, brai and placenta of maternal rats and the blood and brain of foetuses were collected and analyzed for morphine content. The morphine concentration in the blood of maternal and foetal rats was maximal after infusions for 3 hrs; at the maximum, the maternal concentration was 2800 ng/ml and the foetal concentration was 1900 ng/ml. The morphine levels of the brain were also maximal at 3 hrs. The foetal level, 2200 ng/g, was not significantly ($P > 0.05$) different from that of foetal blood, whereas the maternal brain level, 600 ng/g, was less than one-fourth of the maternal blood level. The highest concentration of morphine in the placenta, 6200 ng/g, was also attained after infusion for 3 hrs. These data indicate that the placenta acts as barrier to the passage of morphine from the maternal nimal to the foetus and that blood brain barrier to morphine exists in the adult but not in the foetal rat. Similar studies showed that an effective blood brain barrier is not present in 20 day old rats. Tolerance to the analgesic effect of morphine, 10 mg/kg subcutaneously was demonstrated in non-pregnant adult female rats 2 to 3 days following infusion of morphine, 5 mg/kg per hr for 4 hrs. Reaction times to nociceptive stimulus were thus significantly reduced at 1 hr after the test dose (from 19.6 sec. ± 2.9 S.E.M. in untreated control animals to 6.8 sec ± 0.4 S.E.M. in the morphine infused animals). The saline infused rats had reaction times after the injection of morphine which did not differ significantly from the untreated control rats. Approximately 20 days after the infusion of morphine to day 21 22 pregnant rats the reaction times to test dose of morphine were no longer significantly different from those of control rats given the test dose of morphine. The offspring of morphine infused rats were then tested for analgesic response t morphine, 0.45 mg/kg subcutaneously t approximately 12 days of age and compared with offspring of saline infused rats. The reaction times for offspring of the morphine infused rats 1 hr after the test dose of morphine (26.2 sec. ± 1.1 S.E.M.) were significantly greater than those of the offspring of saline infused rats (20.9 sec. ± 1.5 S.E.M.). A rapid method for preparing isolated nuclei from adult and foetal rat brain in high yield, using detergent treatment and onestep centrifugation on discontinuous sucrose gradient, is described. DNA, RNA and protein content

of homogenates and isolated nuclei from the brains of infused maternal rats and their foetuses were determined. The infusion of morphine over a 4 hrs period did not alter the RNA to DNA ratio or the overall nucleic acid and protein composition of maternal or foetal brain or their isolated nuclei. The amounts of DNA and RNA were lower in the maternal brain and nuclei than in the foetal brain and nuclei. There were higher amounts of protein in the maternal brain than in the foetal brain, whereas in the maternal nuclei there were lower amounts of protein than in the foetal nuclei. It is suggested that morphine may selectively alter RNA and protein synthesis in the brain without producing significant changes in the overall content of either RNA or protein in acute experiments.

Key words: Morphine – pregnancy – foetuses – distribution – analgesia – tolerance – brain – DNA – RNA – protein – nuclei.

In the extensive literature on morphine there are surprisingly few studies pertinent to the distribution of this substance in foetal or neonatal as compared to maternal animals. In the recent literature only the reports of MILTHERS (1960) and KUPFERBERG & WAY (1963) seem to have a bearing on research of this kind as far as rats are concerned. SANNER & WOODS (1965) and BLANE & DOBBS (1967) on the other hand, have studied the distribution of dihydromorphine, a close congener of morphine, in maternal and foetal rats following near term subcutaneous or intramuscular injections.

The experiments reported here were performed in order to investigate the distribution of morphine in maternal and foetal rats following near-term intravenous infusion. COX *et al.* (1968), COX & GINSBURG (1969), and COX & OSMAN (1970) have reported that tolerance to the analgesic action occurs during infusion of morphine in rats and is related to synthesis of RNA and protein in the brain. Therefore, in order to study whether the infusion of morphine in amounts sufficient to induce tolerance in rats produces alterations in the overall composition of either maternal or foetal brain, the amounts of DNA, RNA and protein in maternal and foetal brain homogenates and isolated nuclei were determined.

Methods

Animals.

Adult female Sprague-Dawley (Simonsen) rats weighing about 200 g were housed in stainless steel cages and maintained in constant environmental conditions with free access to tap water and a commercial food preparation (Wayne Lab Blox, Allied Mills, Inc.). The rats were placed with males of demonstrated fertility of the same stock, using one male for two females, from approximately 4:00 p.m. to 8:00 a.m. the next morning. The finding of sperm in vaginal smears was taken as evidence of copulation, this time being designated as the first day (day 1) of pregnancy. The rats were kept in groups of three or four until day 21 or 22 when the appropriate intra-

venous infusion was made. When the rats were allowed to deliver after the infusion, they were kept singly in cages furnished with pans covered with ground corn cobs.

The experiments also included non-pregnant adult female rats, a number of 12 to 13 day old rats, and a group of 20 day old rats.

The studies described here were conducted during July August, September and October 1971.

Infusions.

Infusions were performed on gestational day 21 or 22 (in this stock of rats delivery usually occurs on day 22½). The animals were anaesthetized with ether and tied to an operating table. Incisions were made inside the right hind leg and the femoral vein exposed and cannulated, using No. 50 polyethylene tubing After having inserted the tubing into the vein and ligated it firmly the wound was closed with silk sutures and the animals placed in restraining cages of ppropriate size (No. 90 or 91, Maryland Plastics, Inc.). The animals usually regained consciousness at about the time they were placed in the restraining cage. The infusion pump used (Holter Model RL155) was calibrated to deliver a given volume per hour (usually 1.8–1.9 ml/hr). When morphine was infused, the animals soon became quiet and were often quite catatonic at the time of removal from the cage.

Infusions were made for ½ 1, 2, 3, and 4 hours, respectively At the end of the infusion period the animals were taken from the restraining cage, the tubing clamped and cut, and the animals anaesthetized with ether While under ether anaesthesia, the animals were exsanguinated by heart puncture with heparinized syringes (except for cases where animals were not killed at the end of the infusion periods, see below), the head cut off and the brain (including cerebellum and brain stem) was taken out. Then the abdominal cavity was opened and the foetuses taken out and counted. The foetuses were decapitated, the brain pressed out through the foramen magnum, and as much blood as possible collected from the bleeding neck. Four or five placentas were also taken from each mother Maternal and foetal brains and placentas were cleaned to remove as much external blood as possible, using soft, bsorbent paper and were kept frozen until determinations of morphine were performed few days later Maternal and foetal blood were centrifuged, and the plasma collected and stored frozen until analyzed for morphine. Heparinized syringes and tubes were used for the blood collection and separation. Brains and blood, obtained from foetuses, and the placentas from each mother were, respectively pooled for analysis.

In about one-half of the experiments, the right halves of the maternal brains and half of the brains of foetuses obtained from each mother were collected and immediately placed in small plastic trays which were immersed in an ice bath. The container was then immediately taken to cold room where the brain tissue was weighed and homogenized for determination of DNA, RNA and protein and for preparation of isolated nuclei as described below

The following infusion solutions were used. Saline; morphine, 1 mg/ml (as the base) in saline and morphine, 1 mg/ml, containing 0.5 % 1 % 4 % or 12.5 % radioactive morphine labeled with ^{14}C in the aminomethyl group. The solutions containing radioactive morphine were used in experiments in which the distribution of morphine was studied whereas saline or the non-radioactive solution of morphine in saline were used in experiments on the development of tolerance to the analgesic action of morphine. In the distribution studies, solutions containing the lowest amounts of radioactive morphine were used for the infusions of the longest duration (3 or 4 hours), whereas solutions containing higher amounts of radioactive morphine were

experiments of shorter duration (½, 1 or 2 hours). The dose of morphine (as the base) infused was kept as close to 5 mg/kg/hr as possible and was, with one exception, always in the range of 4.5–5.5 mg/kg/hr.

Radioactive morphine, prepared by the method of ANDERSON & WOODS (1959), was obtained from Amersham/Searle. The specific activity of the compound was 57 mCi/mmol (152 μCi/mg) and radiochemical purity was found to be 96–98 % by four different methods (including paper and thin-layer chromatography). Non-radioactive morphine was Morphine Sulfate U.S.P.

Rats in two groups were infused for four hours with saline and non-radioactive morphine respectively and the animals allowed to deliver naturally. Within 24 hours after delivery the litters were standardized to 8 (4 males and 4 females) when possible. At 1–13 days of age some of the young were tested for the degree of analgesia by a modification of the hot plate method (JÓHANNESSON & BECKER 1972). The remaining young were used for other experimental purposes (STEELE & JÓHANNESSON, to be published). The maternal rats were tested about three weeks after the infusion.

Non-pregnant female rats in two groups were similarly infused for four hours with saline and non-radioactive morphine respectively. Two to three days later they were given a test dose of morphine and the analgesic action measured by the hot plate method of JÓHANNESSON & WOODS (1964).

A number of 10 day old rats of both sexes was injected subcutaneously with 1 mg/kg of morphine (the 1 mg/ml infusion solution containing 4 % radioactive morphine was used). The animals were killed 30 minutes later and morphine was determined in the brains and plasma (brains and plasma from three or four animals were analyzed together). This was done in order to elucidate whether the distribution of morphine in brain and blood was the same as in the foetal rats.

In experiments on the degree of analgesia in the adult animals, morphine was administered by subcutaneous injections given in the middle of the back at the midline. In the young animals injections were made subcutaneously in the neck at the midline. Morphine was dissolved in distilled water and was given in doses of 10 mg/kg (2 ml/kg) subcutaneously to the adult animals and 0.45 mg/kg (10 ml/kg) subcutaneously to the young animals. The doses quoted refer to morphine as the base. The degree of analgesia is expressed as the mean reaction time in seconds ± S.E.M. for a group of animals at 30 and 60 min. after the injection of morphine.

The determination of morphine

The estimation of radioactive morphine in biological materials was essentially the same as that used by JÓHANNESSON & WOODS (1964). However the NH_4OH solution for homogenization was omitted, instead the brains were homogenized in saline. Levels of ^{14}C activity were determined in a Packard Model 3310 Tri-Carb Liquid Scintillation Spectrometer equipped with automatic external standardization. Tissue quench correction and calculation of net disintegrations per minute from the counting rate were determined using a computer programme described by SPRATT & LAGE (1967). The percentage recoveries were consistently about 10 % lower than reported by JÓHANNESSON & WOODS (1964) although the variability of the results was almost the same.

In experiments on the distribution of morphine the mixed drug (labeled and

non-labeled) was, as mentioned before, administered to the animals. Appropriate multiplication factors were therefore used to calculate the total amounts of morphine in the brains, plasma and placenta. The results (ng/g or ng/ml) refer to the total amounts of free or unconjugated drug only

Preparation of isolated nuclei from maternal and foetal brain.

Brain tissues were obtained as described above, transferred to a coldroom (2°) and weighed. Both foetal and maternal brain tissues (bout 1 g) were homogenized I 5 ml of 0.34 M sucrose containing 5 mM tris HCl buffer (pH 8) and 5 mM magnesium acetate (TM) in a glass vessel with a teflon pestle (6–8 × 10^{-3} inch clearance, rotating at 1300 r.p.m.) with 8 strokes for foetal brain and 12 strokes for maternal brain. Aliquots were removed for the determination of DNA, RNA and protein content in whole brain. Triton X 100 (0.38 ml of 10 % solution) was added to the homogenate (5 ml) and mixed by homogenization as described bove with 3 strokes for both foetal and maternal tissues. A portion of the homogenate (5 ml) was transferred to cellulose nitrate centrifuge tube (½ × 4 inch) containing 10 ml of solution of 68 % (w/w) sucrose-TM and mixed vigorously by hand. The mixture was underlayered with a solution of 60 % (w/w) sucrose TM (2.6 ml) and centrifuged (SW 27.1 rotor Beckman Instruments) without delay at 27,000 .p.m. for 1 hr The supernatant solution was decanted and discarded. The tube was inverted and allowed to drain for about 5 min. Cytoplasmic fragments and residual sucrose adhering to the walls of the tube were removed with cellulose wipes while the tube remained inverted. The pellet of isolated nuclei was gently suspended in TM (2 ml) with a loosely-fitting teflon pestle. Aliquots of the nuclear suspension were removed for the determination of DNA, RNA and protein content.

This isolation procedure required less than 90 min. to complete. The duration of contact of exposed nuclei with Triton X 100 never exceeded 10 min. prior to the time of centrifugation (LØVTRUP-REIN & MCEWEN, 1966) Omission of Triton X 100 from the homogenate resulted in gelatinous sediment on centrifugation that contained markedly reduced yield of nuclei. The yield of isolated nuclei, which was calculated on the basis of homogenate DNA and based on the assumption that all cellula DNA is contained within the nucleus, was generally 55–60 % for maternal brain and 80–85 % for foetal brain, but was frequently 70 % and 90 % respectively when a more dilut homogenate was used. The isolated nuclei showed no tendency to aggregate in the TM medium at 0

Determination of nucleic acids and protein in whole brain nd in isolated nuclei.

Samples of brain homogenates and isolated nuclei were precipitated by the addition of 10 volumes of 10 % (w/w) trichloroacetic acid. After standing for 10 min. at 0 the precipitates were pelleted by centrifugation at 5000 × g for 5 min. and the supernatant solutions were decanted and discarded. Th pellets were washed twice with ice-cold 0.5 N perchloric acid by homogenization with a loosely-fitting teflon pestle and centrifuged as before. The RNA content of the pellets was determined according t the method described by FLECK & MUNRO (1962). DNA was determined by modification f the diphenylamine reaction described by BURTON (1956) and protein was determined by the method of LOWRY et al. (1951).

Statistics

The t-test was performed as described by GOLDSTEIN (1964) The level of significance was $P \leq 0.05$.

Results

Distribution studies

As previously mentioned, each rat was infused with morphine in doses which were in the range 4.5–5.5 mg/kg/hr The results obtained from any particular rat were subsequently standardized to the mean dose, 5.0 mg/kg/hr

The amount of infused morphine in the maternal plasma rose and quickly reached a maximum at three hours, but was considerably lower at 4 hours. The concentration was thus not much different after 2 or 4 hours of infusion (fig. 1) In maternal brain, the morphine concentration was always 4 to 5 times lower than in the plasma, but the rise and decline of the morphine concentration in brain showed the same pattern as was observed in plasma (fig. 1)

Fig. 2 shows the results of morphine determinations in the brain and plasma of foetal rats. The rise and fall with time of the amounts was similar to that seen in the maternal animals (fig. 1) Unlike adults, morphine was always found in greater amounts in foetal brain than in foetal plasma, although this difference was not statistically significant. The amounts of morphine in the foetal brain were on the whole, about three times those found in maternal brain Morphine was, on the other hand, always found in greater

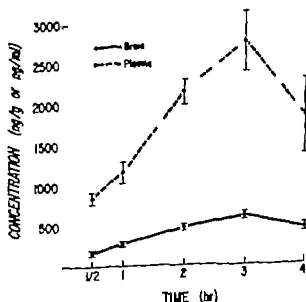


Fig. 1. The concentration of morphine in maternal brain and plasma (ng/g ng/ml) at ½ 1 2, 3 and 4 hours after intravenous infusion of morphine (5 mg/kg/hr). Each value is the mean value [illegible] with 5–9 rats. The vertical lines indicate the s[illegible] of the mean (S.E.M.).

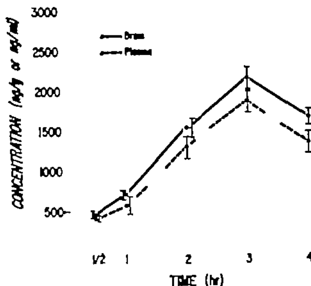


Fig. 2. The concentration of morphine in foetal brain and plasma (ng/g; g/ml) at ½ 1 2, 3 and 4 hours after intravenous infusion of morphine (5 mg/kg/hr). See fig 1.

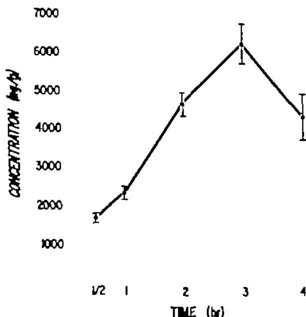


Fig. 3. The concentration of morphine in placenta (ng/g) at ½ 1 2, 3 and 4 hours after intravenous infusion of morphine (5 mg/kg/hr). See fig. 1.

amounts in the maternal than in the foetal plasma. The difference was statistically significant at ½ 1 2 and 3 hours, but not at 4 hours.

The amounts of morphine in the placentas are shown in fig. 3 The concentration of morphine in the placenta was very great and followed the same time pattern as in brain and plasma. Placental concentrations were significantly different at all time periods from those of both maternal and foetal plasma At 3 hours morphine was found in excess of 6,000 ng/g in the placenta and at 2 and 4 hrs the concentration was between 4,000 and 4,500 ng/g.

Sixteen 20 day old rats of both sexes were injected with morphine 1 mg/kg subcutaneously and killed 30 minutes later The average brain concentration of morphine was 81 ng/g. In plasma the average concentration was 94 ng/ml. The difference was not statistically significant.

Analgesimetry

Adult female non-pregnant rats in two groups of 6 each were infused for 4 hours with saline or morphine, 4 90–5 40 mg/kg/hr respectively Two to three days later they were injected with morphine, 10 mg/kg subcutaneously and the analgesic action measured. A group of untreated adult female rats was similarly treated. The following results were obtained (the first values denote the mean analgesic action in seconds ± S.E.M at 30 minutes and the latter ones at 60 minutes after injection)·

saline – 20 0 sec. ± 3 7 22 1 ± 3 7
morphine – 6.5 sec. ± 1.0, 6.8 ± 0 4
untreated animals – 17 1 sec. ± 2.3 19 6 ± 2.9

The analgesic action in the morphine treated rats was obviously lower than that in the saline treated rats. The degree of analgesia observed in the saline infused and the untreated animals was of the same order of magnitude.

Pregnant rats in two groups, 8 and 9 in each were infused on day 21 or 22 for 4 hours with saline or morphine, 4 70–5 45 mg/kg/hr The rats were allowed to deliver naturally and were tested for analgesic activity 18–21 days later following a single subcutaneous dose of morphine A third group of non-pregnant rats, 8 in number served as untreated controls. Rats in all groups were injected subcutaneously with morphine, 10 mg/kg.

The results are shown below (the values denote the mean analgesic action in seconds ± S.E.M at 30 minutes and at 60 minutes after injection in that order)

saline – 15 8 sec. ± 2.8, 22.9 ± 3.5
morphine – 11.8 sec. ± 3.5 16.3 ± 3 7
untreated controls – 15 6 sec. ± 2.8 24.3 ± 3 7

The analgesic action was not significantly different in these groups. (In this connection it should be mentioned that two rats in the morphine pretreated group responded with maximal analgesic action (30 sec.) both at 30 and 60 min., whereas the analgesic action was generally much lower in the other rats in this group.) The mean weight of the saline infused rats was significantly higher than that of rats in the morphine treated group.

Two groups of 12–13 day old rats were given a test dose of morphine, 0.45 mg/kg subcutaneously and the analgesic action was measured. There were 30 animals in each group one consisting of young of mothers infused with morphine and the other of mothers infused with saline. In the morphine treated group the mean analgesic action measured in seconds ± S.E.M. was 22.6 ± 1 6 at 30 min. and 26.2 ± 1.1 at 60 min., whereas in the saline group the respective values were 17.2 ± 1 6 and 20 9 ± 1.5 The differences in analgesic action in these two groups were found to be statistically significant

Even though animals in both groups were from standardized litters (cf Methods) the offspring of maternal rats infused with morphine had a mean body weight (22.2 g ± 0.6 S.E.M.) which was significantly lower than that of offspring of maternal rats infused with saline (25.3 g ± 0.6 S.E.M)

DNA RNA and protein in brain and isolated brain nuclei

The nucleic acid and protein composition of foetal brains and isolated nuclei of foetal brains from untreated and morphine infused day 21–22 pregnant rats is presented in table 1 Neither the ratio of RNA to DNA nor the overall amounts of DNA, RNA and protein in foetal brain or isolated nuclei of foetal brain cells were significantly altered after short and long-term infusions of morphine, respectively

The nucleic acid and protein composition of brain and isolated nuclei of brain from untreated and morphine infused day 21–22 pregnant rats is presented in table 2. Neither the ratio of RNA to DNA nor the overall amounts of DNA, RNA and protein in maternal brain or isolated nuclei of maternal brain were significantly altered after short and long-term infusions of morphine, respectively

The greatest difference in chemical composition between foetal and maternal brain (tables 1 and 2) was found to be a threefold greater amount of protein relative to DNA in maternal brain than in foetal brain. Isolated nuclei of foetal brain contained a greater percentage of the cellular RNA and protein, 25 / and 28 / respectively than isolated nuclei of maternal brain, which contained only 18 / and 8 / respectively of the total cellular RNA and protein. The brains of maternal rats (about 2 g) contained about a five fold higher number of cells (mg DNA) than the brains of day 21–22 foetuses (about 0.2 g) assuming that the DNA content of brain is directly proportional to the number of diploid cells.

Table 1

Nucleic acid and protein composition of foetal whole brain and isolated nuclei of foetal brain from untreated and morphine infused (5 mg/kg/hr) day 21–22 pregnant rats. Results are given as the mean ± S.E.M. of values from 3–9 experiments. Values for the composition of isolated nuclei were calculated on the basis of the recovery of DNA in isolated nuclei (80–85 %). Data from short-term, ½ and 1 hr and long-term, 3 and 4 hrs, infusions were pooled.

Fraction	Duration of infusion	DNA	RNA	Protein	RNA/DNA	Protein/DNA
Whole brain	none	3.06 ± 0.08	3.62 ± 0.03	61.2 ± 2.2	1.17 ± 0.03	20.5 ± 0.8
	½ and 1 hr	3.18 ± 0.05	3.93 ± 0.06	N.D	1.23 ± 0.01	–
	3 and 4 hrs	3.21 ± 0.06	3.61 ± 0.10	57.5 ± 2.1	1.13 ± 0.04	18.0 ± 0.6
Isolated nuclei	none	3.06 ± 0.08	0.93 ± 0.09	17.1 ± 0.7	0.30 ± 0.02	5.6 ± 0.1
	½ and 1 hr	3.18 ± 0.05	1.01 ± 0.05	N.D	0.32 ± 0.01	–
	3 and 4 hrs	3.21 ± 0.03	0.93 ± 0.04	17.8 ± 0.4	0.30 ± 0.00	5.5 ± 0.1

Expressed as mg/g wet weight of brain.
** N.D not determined.

Table 2

Nucleic acid and protein composition of maternal whole brain and isolated brain nuclei from untreated and morphine infused (5 mg/kg/hr) day 21 22 pregnant rats. Results are given as the mean ± S.E.M. of values from 3–9 experiments. Values for the composition of isolated nuclei were calculated on the basis of the recovery of DNA in isolated nuclei (55–60 %). Data from short-term, ½ and 1 hr and long-term, 3 and 4 hrs, infusions were pooled.

Fraction	Duration of infusion	DNA	RNA	Protein	RNA/DNA	Protein/DNA
Whole brain	none	1.47 ± 0.07	1.89 ± 0.04	97.7 ± 3.9	1.29 ± 0.05	67.0 ± 5.4
	½ and 1 hr	1.43 ± 0.04	1.82 ± 0.03	N.D.	1.27 ± 0.04	–
	3 and 4 hrs	1.47 ± 0.04	1.81 ± 0.04	90.4 ± 2.9	1.24 ± 0.02	62.4 ± 1.7
Isolated nuclei	none	1.47 ± 0.07	0.35 ± 0.02	7.7 ± 0.4	0.24 ± 0.00	5.2 ± 0.1
	½ and 1 hr	1.43 ± 0.04	0.36 ± 0.01	N.D.	0.25 ± 0.01	–
	3 and 4 hrs	1.46 ± 0.05	0.37 ± 0.01	7.8 ± 0.4	0.26 ± 0.01	5.3 ± 0.3

[illegible] weight of brain.
N.D. [illegible]

The RNA to DNA and protein to DNA ratios for isolated nuclei of maternal brain (table 2) are of the same order as those obtained by LØVTRUP-REIN & MCEWEN (1966) for adult rat brain. The amounts of DNA, RNA and protein in the brains of maternal rats and day 21–22 foetal rats reported here are in close agreement with the results of other workers (WINICK & NOBLE 1965, CLOUET & GAITONDE 1956).

Discussion

Infusion of morphine into pregnant rats over a four hour period caused the concentration of morphine to increase with a peak at 3 hours and then decline in all the tissues examined. Thus, the tissue levels of morphine were nearly the same with infusion at 2 and 4 hours (fig. 1–3). This may represent the interplay of various biological factors, such as patterns of permeability and distribution in blood and other tissues, metabolism in the liver and the extent of elimination.

In this connection the results of COX *et al.* (1968) are of considerable interest. These workers infused morphine into rats at a rate of 5–10 mg/kg/hr and tested the reaction of the animals to a painful stimulus (mechanical pressure on the tail) during the infusion period. They were able to discern three phases in the analgesic response to morphine. A phase of increasing analgesia of 90–120 min. in duration, followed by a phase in which a peak analgesia was maintained for another 90–120 min. and finally a phase of decline. After infusion for 8 hours the analgesic response was almost minimal. COX *et al.* ascribed the decline of the analgesic response to the development of tolerance to the action of morphine. Although there is little doubt that tolerance develops after infusion of morphine (see below), it seems likely that the rise and fall in the analgesic action of morphine corresponds closely to the rise and decline in the concentration of morphine in the brain as observed in our experiments. Moreover, this is born out by other experiments of COX *et al.* (1968), in which they repeatedly infused morphine into the same animals and still observed the same pattern of analgesic response, even though the analgesic action of morphine gradually declined (cf. their fig. 8).

The morphine content of the placenta was always significantly higher than that found in the maternal or foetal plasma. Morphine was, however, consistently found in greater amounts in maternal than in foetal plasma (figs. 1 and 2). Thus, the placenta appears to act as a barrier to the passage of morphine from maternal to foetal plasma. Moreover, the finding of very high amounts of morphine in the placenta would suggest that this organ may have the ability to retain morphine. The results of GAUTIERI & CIUCHTA (1962) are of particular interest in this connection. In studies on human placentas, they found that perfusion with morphine reduced the volume of outflow by 35–

90 / and concluded that morphine may exert a direct vasoconstrictor effect on placental vessels. This was supported by the fact that the administration of nalorphine prevented the action of morphine and almost returned the flow to normal. Thus, it is likely that administration of morphine may result in the pooling of blood in the placenta, which could explain to some extent the very high amounts of morphine found in this organ (fig. 3)

The results of our experiments show that morphine was present in much greater amounts in the foetal than in the maternal brain Morphine was, moreover found in greater amounts in the foetal brain than in the foetal plasma, although the difference was not statistically significant. This is in sharp contrast to the phenomena observed in maternal rats (figs. 1 and 2) Thus, the so-called brain-blood barrier must be much less effective or developed in foetal than in maternal rats. This is in agreement with the results of SANNER & WOODS (1965) and BLANE & DOBBS (1967) who studied the distribution of dihydromorphine in maternal and foetal rats following subcutaneous or intramuscular injections.

In studies on the infusion of morphine into adult and 12 day old rats, MILTHERS (1960) has shown that the amounts of morphine (both absolute and relative to the amounts in blood) were much greater at the time of death in the brains of the young animals than in the brains of the adult animals. The results of our experiments with subcutaneous injections of morphine to 20 day old rats indicate that the distribution of morphine in these animals is not very different from that in the foetal rats (see Results) The results of KUPFERBERG & WAY (1963) with intraperitoneal injections of morphine (50 mg/kg) to young rats showed that much greater amounts were found in the brains of 16 day old rats than in the brains of 32 day old rats. In summary the above mentioned deficiency in the brain-blood barrier in foetal rats, as far as morphine is concerned, is still present to a large extent at the age of 3 weeks, but thereafter probably soon disappears.

Tolerance to the analgesic action of morphine was demonstrated when non-pregnant rats were tested 2–3 days after the infusion of morphine (total dose was 20 mg/kg/4 hrs), whereas pregnant rats, treated in the same way and tested 3 weeks after delivery failed to show a significant degree of tolerance. This is in contrast to the results of JÓHANNESSON & BECKER (1972) who found a high degree of tolerance present at 4 weeks after delivery in rats that had been given four daily subcutaneous injections of morphine (20 mg/kg/day) in the last week of pregnancy These results indicate that among other factors, the route and the number of doses given may determine how long tolerance persists.

JÓHANNESSON & BECKER (1972) found that 12–13 day old young of the abovementioned mothers that had been injected with morphine late in pregnancy were tolerant to the analgesic action of morphine. This

not the case with 12–13 day old young of mothers infused intravenously with morphine On the contrary the young of morphine infused mothers showed a significantly higher degree of analgesia than the young of saline infused mothers. There is no ready explanation for this phenomenon of apparent hypersensitivity to the analgesic action of morphine. However hypersensitivity may have followed an undetected and short lived tolerance.

Cox *et al* (1968) reported that simultaneous infusion of actinomycin D (10 µg/kg/hr) and morphine prolonged the peak of maximal analgesia and delayed its decline (see above). Actinomycin D had no analgesic effect of its own (their fig. 4). More recently they showed that actinomycin D apparently has no effect on the concentration of morphine in the brain (Cox & Ginsburg 1969) and that a number of other inhibitors of RNA and protein synthesis could modify the analgesic response in a manner similar to that of actinomycin D (Cox & Osman 1970). These substances were more effective when given by intracerebral injections than when given intravenously Cox & Osman (1968) concluded on the basis of their experiments that substances like actinomycin D hinder the development of tolerance to the analgesic action of morphine and that this effect is causally related to the inhibition of RNA and protein synthesis. Quite apart from the question of whether or not the phase of declining analgesic effect of morphine as observed in their experiments, denotes development of tolerance, it seems extremely difficult to explain in explicit terms the effects of substances like actinomycin D which may profoundly alter the function of most cells in the body At any rate, infusion of morphine in amounts high enough to induce tolerance does not result in any clear gross changes in the content of DNA, RNA and protein in the brains or isolated nuclei of maternal and foetal rats (tables 1 and 2). The amounts of DNA, RNA and protein were essentially the same after infusion for ½ and 1 hour as after infusion for 3 and 4 hours. On the other hand, the offspring of morphine treated mothers gained weight at a slower rate than the offspring of non-treated mothers and even the treated maternal rats gained weight more slowly than the corresponding control rats (cf. Results). Morphine thus appears to interfere with the synthesis of protein in the body

In this connection inhibitory effects of morphine and morphine congeners on the growth of microorganisms and mammalian cells in culture should be mentioned. In Hela cells, these effects have been correlated with an inhibition of RNA and protein synthesis (Noteboom & Mueller 1969), the latter being associated with polysome breakdown. Polysome instability has also been found in adult rat brain after intraperitoneal administration of a single dose of morphine (Clouet 1971) and in foetal brain after the infusion of morphine over a 4 hrs period (Steele & Jóhannesson to be published). Although it is obvious that the infusion of morphine as described here does not result in any gross changes in the amounts of DNA, RNA and protein in

the maternal or the foetal brains, it is still possible that morphine could selectively alter RNA and protein synthesis in the brain without causing a significant change in the overall content of either RNA or protein in acute experiments.

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From Gist Brocades N V Research & Development,
Parklaan 125, Haarlem, The Netherlands

Differences in Metabolic Behaviour and Liver Toxicity Between the Optical Isomers of Bufenadrine Hydrochloride, a Substituted Diphenhydramine, in the Rat

By

W Hespe, D. Mulder and C. J van Eeken

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Abstract Among the alkyl-substituted amino ethers derived from diphenhydramine, bufenadrine, 2-[(*o-tert*-butyl-α-phenylbenzyl)oxy]-*N*,*N*-dimethylethylamine, is a remarkable exampl of the role of structural factors in liver toxicity Non-published toxicological investigations with both optical isomers of this compound revealed that (−)bufenadrine is exclusively or mainly responsible for the toxic activities of the racemic compound. Metabolic studies in rats with both optical isomers labelled N-$^{14}CH_3$ showed that after a single dose profound differences already occur with regard to their metabolic fate. Over the period studied the elimination of radioactivity after dose of both 2 mg/kg, intravenously and of 10 mg/kg, orally of the (−)isomer was lower in the urine, faeces and bile and higher in the respiratory air than with the (+)isomer When an oral dose of 50 mg/kg was given, the elimination in the respiratory air too, was lowest in the case of the (−)isomer Since no significant difference in binding to serum constituents or to homogenated liver tissue was observed, it seems likely that the metabolic differences originate in the liver The data obtained suggest the inability of the liver to metabolize (−)bufenadrine at rate sufficient to prevent the accumulation of the substance which triggers off the toxic mechanism.

Key-words: Bufenadrine – *N*-demethylation – diphenhydramine derivative – labelled optical isomers – liver toxicity – metabolic fate.

It is well established that compounds – even though structurally closely related – may differ considerably in their action on the organism. The influence of drugs on the liver seems to be no exception. A remarkable illustration of the part played by structural factors in liver toxicity was afforded by bufenadrine (2-[(*o-tert*-butyl-α-phenylbenzyl)oxy]-*N*,*N*-dimethylethylamine, WHO) hydrochloride (fig. 1) Because of its interesting pharma cological properties this compound was originally intended for

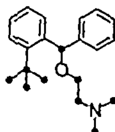


Fig. 1. Bufenadrine.

anti-motion sickness drug (HARMS *et al* 1962). Prolonged toxicity studies in rats however showed liver toxicity and no further clinical evaluation was therefore undertaken The toxic phenomena observed in the rat of all dosage groups (7 14 28 mg/kg orally) and so far unpublished included liver abnormalities such as fatty infiltration, vacuolization of the liver parenchyma, polymorphism, an unusually high number of mitoses (double-nucleated cells) and lymphocytic infiltration. In addition, both male and female rats showed growth retardation as well as a marked increase in erythrocytes in the highest dose group After a 10-day recovery period no regeneration appeared to have occurred in the liver lesions on the contrary the degree of fatty infiltration increased, particularly in the lowest dose group Continued toxicity studies with both the optical isomers of bufenadrine hydrochloride which were separated by our Chemistry Department according to a procedure described by HARMS (1956), strongly suggested that the (−)isomer with the stronger anticholinergic and the weaker anti-histaminic activities is mainly responsible for the toxicity of the racemic compound (unpublished results of our Toxicology Department).

The great difference in the toxicologic properties between the two optical isomers of bufenadrine hydrochloride prompted us to investigate the metabolic fate of both compounds, it being assumed that differences in metabolic fate might be among the underlying causes of the discrepancy in the toxicity

Although the toxicity of the racemic bufenadrine hydrochloride or its (−)isomer only became manifest in prolonged toxicity studies, we preferred to start with single doses of either isomer Our first aim was to look for a metabolic parameter for liver toxicity at a time when the increasing liver damage had not yet begun to affect the metabolism of the drug.

Material and Methods

^{14}C-labelled bufenadrine hydrochloride.

The metabolic studies were performed with both bufenadrine isomers in N-$^{14}CH_3$

labelled form, synthesized by our Tracer Laboratory. The specific radioactivity of both isomers was 1.5 mCi/g (0.5 mCi/mmol). Chemical and radiochemical purity were established by thin-layer chromatography.

Animals.

In all the experiments male rats of the TNO-Wistar CPW/Wu strain, in SPF condition, were used; each animal weighed about 180 g.

Excretion experiments.

The radioactive substances were administered intravenously to non-fasted rats as a solution in physiological saline and orally as a solution in water to rats, fasted for 20 hrs.

The excretion of radioactivity in the urine, faeces, and expired carbon dioxide was determined over subsequent time intervals, metabolic cages being used for the separate collection of these excretion products.

To study the excretion in bile a polyethylene cannula was inserted into the common bile duct of rats, under phenobarbital (phenemalum NFN) sodium anaesthesia (100 mg/kg, intraperitoneally) and bile was continuously collected over periods up to 5 hrs after the administration of the labelled compounds.

Binding experiments.

In order to study the binding of both labelled bufenadrine isomers to blood and liver constituents, 1 ml of rat serum or rat liver homogenate (corresponding to 100 mg of liver tissue) and 20 ml of buffer solution, pH = 7 (0.1 mol of citrate-phosphate) were mixed in a dialysis bag (Visking 27/32). The bag was immersed in buffer solution containing 6 µg/ml of one of the labelled isomers. After standing for 24 hrs at 37° the radioactivity of the solution in both compartments was determined and the percentage binding calculated, according to:

$$\text{binding} = \frac{\text{DPM per ml in dialysis bag} - \text{DPM per ml in dialysis bath}}{\text{DPM per ml in dialysis bag}} \times 100$$

(DPM = disintegrations per minute)

Analytical studies.

The urine was analyzed for unchanged bufenadrine and radioactive metabolites by a combined extraction/TLC procedure.

First the urine was adjusted to pH = 12 and extracted with heptane and butanol. The residue was adjusted to pH = 4.5 and treated with β-glucuronidase/arylsulfatase (Sigma) at 37° for 24 hrs. A second extraction with butanol at pH = 12 was then performed.

To allow chromatographic analysis the extracts were evaporated (Rotavapor), each residue taken up in a small volume of ethanol, and the resultant solutions spotted on TLC plates covered with Silicagel G (Merck).

As solvent system butanol-ammonia (98:2) was used. In this system bufenadrine has an R_f value of about 0.70 and *N*-demethyl bufenadrine, the only potential metabolite we had available for comparative purposes, an R_f of 0.43.

In order to visualize these products Dragendorff's reagent was used.

Non-radioactive bufenadrine and *N*-demethyl bufenadrine were used as internal markers (added to the solution to be spotted), in order to facilitate interpretation of the chromatograms.

Table I

Elimination pattern of the radioactivity in male rats after a dose of (+) or (−)bufenadrine hydrochloride-N-$^{14}CH_3$

Isomer	Route of administration	Dose (mg/kg)	Mean elimination as a % of the administered radioactivity: urine (0–72 hrs)	faeces (0–72 hrs)	$^{14}CO_2$ (0–7 hrs)	sum	bile (0–5 hrs)
(+)	i.v	2	30.3 ± 8.7 (5)	34.1 ± 12.0 (5)	26.3 ± 5.9 (5)	90.7	37.9 ± 2.2 (4)
(−)	i.v	2	15.8 ± 2.9 (7)*	15.7 ± 5.0 (7)	48.8 ± 7.4 (7)*	80.3	19.2 ± 1.1 (4)*
(+)	or	10	19.3 ± 1.3 (4)	28.0 ± 5.0 (4)	31.3 ± 2.1 (5)	78.6	20.7 ± 4.5 (4)
(−)	or	10	15.0 ± 2.0 (3)*	18.6 ± 6.8 (3)	40.4 ± 5.3 (4)	74.0	4.7 ± 2.2 (4)
(+)	or	50	21.7 ± 0.1 (2)	13.6 ± 2.8 (2)	32.1 ± 2.2 (2)	67.4	–
(−)	or	50	14.7 ± 1.2 (2)*	17.6 ± 4.0 (2)	20.3 ± 0.3 (2)	52.6	–

* According to the Student's t-test significantly different from the (+)isomer ($P < 0.05$).

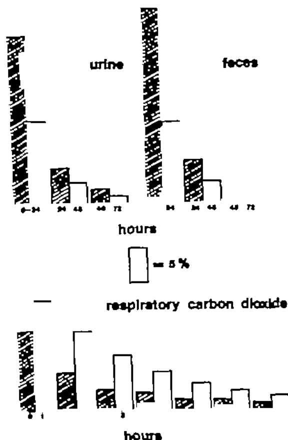


Fig. 2. The excretion of radioactivity in the urine, faeces and respiratory air after intravenous administration of 2 mg/kg of labelled (+)bufenadrine hydrochloride (shaded bars) and (−)bufenadrine hydrochloride (open bars) to rats. The values plotted are the mean of the results obtained in 4 rats.

Radioactivity measurements.

Samples of urine, bil and extracts (0.5 ml) were mixed with 10 ml of scintillation mixture according to BRAY (1960) in plastic counting vials.

The expired carbon dioxide was bound into an ethanolamine-containing scintillation mixture (JEFFAY & ALVAREZ 1961), of which 15 ml aliquots were measured.

The radioactivity in the faeces was determined according to the method of MAHIN & LOFBERG (1966), samples of the material to be measured being destructed direct in counting vials. Th scintillation cocktail was then added.

The counting of the various samples was performed by means of a Packard Tri Carb Scintillation Spectrometer 3375, using the method of external standardization.

Thin-layer chromatograms were scanned for radioactive spots using a Berthold Dunnschicht Scanner LB 2720 with a combined integrator

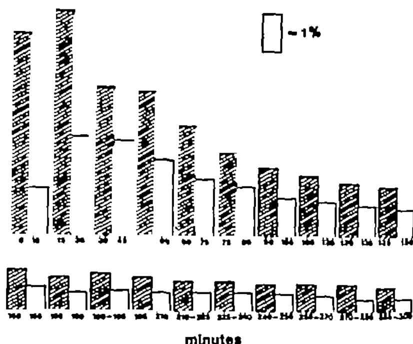


Fig. 3 The excretion of radioactivity in bile after intravenous administration of 2 mg/kg of labelled (+) bufenadrine hydrochloride (shaded bars) and labelled (−)bufenadrine hydrochloride (open bars) to bile fistula rats. The values plotted are the mean of the results obtained in 4 rats.

Results

Excretion experiments.

Table 1 lists the overall results of the excretion experiments in rats that had received single doses of either labelled isomer of bufenadrine hydrochloride

Both intravenous and oral dose produced significant differences between *the elimination patterns of the isomers.*

As for the intravenous and the lower oral dose, the elimination of radioactivity from the (−)isomer was, as compared to the (+)isomer lower in the urine, faeces and bile and higher in the expiratory air over the period studied.

As regards the higher oral dose the elimination of the radioactivity from the (−)isomer compared to the (+)isomer was lower in the urine and the expiratory air no obvious difference being observed in the percentages of faecal elimination.

Qualitatively the overall differences shown in table 1 persisted at nearly

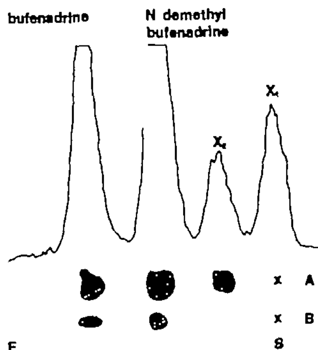


Fig. 4 Thin-layer chromatogram (A) and corresponding radioactivity distribution of heptane extract of rat urine, collected over 24 hrs after oral administration of 50 mg/kg of (−)bufenadrine hydrochloride-N-$^{14}CH_3$. Non-radioactive bufenadrine hydrochloride and *N*-demethyl bufenadrine hydrochloride were added as internal markers. Chromatogram B shows both compounds as external reference substances. The shaded spots became visible after treatment with Dragendorff's reagent.

all the time intervals studied. Figs. 2 and 3 illustrate this for the 2 mg/kg intravenous dose.

Analysis of the urine.

TLC of extracts of urine of rats dosed with either labelled isomer showed a good separation of several radioactive products, including the intact product and the *N*-demethylated derivative (see fig. 4) In each extract there were several unknown products.

Qualitatively the same spectrum of metabolites was observed for both bufenadrine isomers, but quantitatively distinct differences were found as illustrated in table 2.

The metabolites not yet identified are indicated by a letter which is different for each type of extraction, although several of these unknown products found in the various extracts are probably identical.

Table 2

Chromatographic analysis of urine, collected over a 4 hr-period after oral administration of 50 mg/kg of (+) or (−)bufenadrine hydrochloride N-$^{14}CH_3$ to male rats*. Each figure represents the mean of data obtained in 2 rats.

Successive treatments of the urine	Radioactive products in extract	Approximate radioactive R_f value	Amounts extracted (µg equivalents of bufenadrine hydrochloride) (+)	(−)
Heptane extraction	X_1	0.00	6	3
	X_2	0.20	30	12
	N-demethyl bufenadrine	0.43	3	16
	bufenadrine	0.70	2	11
Butanol extraction	Y_1	0.00	346	59
	Y_2	0.14	474	178
	Y_3	0.30	55	42
	Y_4	0.36	36	32
Butanol extraction after enzymatic treatment	Z_1	0.00	7	1
	Z_2	0.06	56	17
	Z_3	0.24	56	16
	Z_4	0.43	13	6
	Z_5	0.49	4	5
		Sum	1088	398
		Amount originally present in urine	1542	596
		Yield of the extraction procedure	71 %	67 %

Separate experiment from that with an equal dose, mentioned in table 1

The total amount of radioactive material accounted for in the urine in the case of the (+)isomer was much higher than with the (−)isomer, a difference which is ascribed to the greater excretion of metabolites, particularly Y_1 and Y_2, after the administration of (+)bufenadrine hydrochloride. The only two products predominating in the case of the (−)isomer were the intact substance and its N-demethylated derivative. Although some of the products showed a less perfect separation, the chromatographic analysis of urine after an oral dose of 10 mg/kg was essentially the same and allows the same conclusion.

Binding studies.

The binding of both radioactive bufenadrine isomers to serum and liver constituents was studied in dialysis experiments, the results of which are listed in table 3.

Table 3

Binding of (+) and (−)bufenadrine hydrochloride-N-$^{14}CH_3$ to constituents of rat liver and serum as determined in dialysis experiments.

Isomer	Percentage bound	
	Serum	Liver homogenate
(+)	18.9 ± 1.5 (5)	42.2 ± 2.9 (5)
(−)	16.6 ± 2.8 (5)	37.6 ± 1.6 (5)

Discussion

Differences in metabolic fate between optical isomers of drugs have been repeatedly reported in the literature. For example, (−)pentobarbital (mebumalum NFN) is inactivated more rapidly in the rat than its antipode which consequently reaches higher levels in the organism (BÜCH *et al.* 1969). The two isomers of chloramphenicol differ markedly in the excretion rate in rat bile (GLAZKO 1965). Labelled (−)benzetimide was found to be excreted more rapidly in the rat urine than the (+)isomer (VAN WIJNGAARDEN 1969). These and other examples reported in the literature suggest that differences in metabolic behaviour of optical isomers are a common feature, as is to be expected from the asymmetrical structure of those macromolecules which interact with the drug. Our study with bufenadrine confirms this.

One obvious explanation for the differences observed in the elimination patterns of bufenadrine isomers at the lower doses studied is depicted in fig 5. It is suggested that there is competition between *N*-demethylation on the one hand and biotransformation to hydrophilic products – presumably aromatic hydroxylation, followed by conjugation – on the other. The individual elimination patterns of both isomers occur because (1) the (−)isomer is less rapidly transformed into hydrophilic products which are readily excreted in bile or urine and/or (2) the (+)isomer is less rapidly *N*-demethylated.

At the 50 mg/kg dose level the elimination percentage of the radioactivity of the (−)bufenadrine hydrochloride in the expired air decreases substantially as compared to the percentages after the lower doses. It even falls below that of the (+)isomer where a dose-dependent effect was not found. Obviously at higher dose levels the *N*-demethylation mechanism also becomes affected by the (−)isomer.

The established differences in metabolic fate between (+) and (−)bufenadrine suggest that biotransformation processes in the liver are major determining factors. An alternative hypothesis, which would at least explain part of the differences in the elimination pattern of both isomers in-

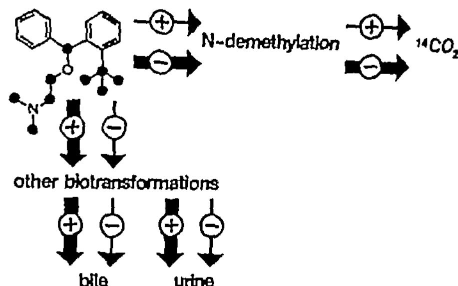


Fig. 5 Possible explanation for differences in metabolic behaviour of the optical isomers of bufenadrine hydrochloride in the rat after a single dose of 2 mg/kg, intravenously or of 10 mg/kg, orally

volves a difference in fate *before* the enzymatic sites concerned in the *biotransformation are reached. Although differences in binding to serum* proteins or in the affinity to liver tissue might be involved, the results obtained (table 3) make this less likely

Under the experimental conditions used the two isomers showed about the same degree of binding and on statistical analysis of the data (Student's t-test) the differences appeared not to be significant ($P > 0.05$). The *differences in kidney function too did not provide an explanation*, since in this case a decrease in the urinary elimination would increase biliary excretion. In the case of the (−)bufenadrine the data show that both the *urinary and biliary excretion are decreased as compared* with the (+)isomer.

The hypothesis that the metabolic differences between both bufenadrine isomers are the result of a slower biotransformation of (−)bufenadrine *is supported by the analytical findings*. These show that the greater amount of (+)bufenadrine radioactivity eliminated in the urine is due to the elimination of a larger quantity of hydrophilic metabolites. As regards the (−)isomer, more of the intact product was recovered from the urine. There was also a larger recovery of the *N*-demethylated product. This shows that once it has been formed, its further biotransformation also proceeds less rapidly in the case of the (−)isomer The slower biotransformation of (−)bufenadrine leads to a comparatively higher accumulation, particularly on repeated administration.

With the 2 mg/kg intravenous dose the percentage of the (−)bufenadrine radioactivity recovered as labelled carbon dioxide amounted to nearly 50 / over the period studied. During *in vitro* studies with liver microsomes (ROOZEMOND *et al* 1965) bufenadrine had already been shown to be an excellent substrate for the *N*-demethylation reaction. Thus if only mono-*N* demethylation occurred it might be concluded that practically all the in travenous (−)bufenadrine had undergone this process, since only one of the $N\text{-}^{14}CH_3$ groups was labelled. In practice, di-*N*-demethylation can also be expected. Since there is substantial *N*-demethylation of (−)bufenadrine at the 50 mg/kg-level it should be noted that among the accumulating products the *N*-demethylated products might also play an important role. The data obtained allow for a relation between the toxicological and metabolic findings. the inability of the rat liver to transform (−)bufenadrine hydrochloride and probably its N-demethylated derivative at a rate suf ficient to prevent the accumulation which triggers off the toxic mechanism.

The (+)isomer and also its related compounds found not to be toxic to the liver may have the intrinsic activity to induce biochemical changes resulting in hepatotoxic manifestations as described above, though in prac tice they do not reach a critical level in the liver tissues, because of a sufficiently rapid biotransformation to hydrophilic products which are readily excreted.

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From the Department of Pharmacology University of Oulu,
90 100 Oulu 10 Finland

The Effect of Phenobarbital Pretreatment on the Cerium Induced Impairment of Drug Metabolism in Rat

By

Pentti Arvela and Niilo T Kärki

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Abstract The effect of phenobarbital pretreatment has been studied on cerium induced impairment of drug metabolism i rat liver Male rats were divided into four groups. (1) controls, (2) receiving cerium intravenously 2 mg/kg, (3) pretreated with phenobarbital and (4) receiving the same amount of cerium as in group after pretreatment with phenobarbital as in group 3 The pretreatment had a clear protective effect both against the cerium induced impairment of oxidative drug metabolism and the elevation of some enzyme activities. The pretreatment also normalized the decrease in blood glucose and the increase in plasma free fatty acids caused by cerium.

Key-words: Rare earths – cerium enzyme induction liver injury

Rare earth metals are known to be toxic elements, helpful agents in medicine and fission products. Previous studies have demonstrated that the liver damage caused by a single intravenous injection of cerium is manifested by the production of fatty liver (SNYDER & STEPHENS 1961), morphological changes both in the liver mitochondria (GLENN *et al.* 1962) and endoplasmic reticulum (MAGNUSSON 1963) and by an inhibitory effect on liver microsomal drug metabolizing enzymes (ARVELA & KÄRKI 1971). The acute liver damage produced by the rare earth metals may also be responsible for the insufficient glycogen formation which in its turn causes hypoglycaemia (MAGNUSSON 1963) Pretreatment which drugs which are known to stimulate the microsomal drug metabolizing enzymes has shown that they are of therapeutic value against various toxic substances both in newborn infants (TROLLE 1968 YAFFE *et al* 1966) and adults (KREEK & SLEISENGER 1968 THOMPSON & WILLIAMS 1967) On the other hand stimulation of liver microsomal enzymes may lead to a more marked effect of some drugs and poisons (MARSHALL & McLEAN 1969 VORNE 1971 HOCHSTRATE & OBERDISSE 1970). In our study

we have investigated whether pretreatment with phenobarbital (phenemalum NFN) which is a proved and commonly used inducing substance, has any effect on the cerium (2 mg/kg) induced impairment of the drug metabolizing system in rats.

Material and methods

In the present study 25 male Sprague-Dawley rats each weighing pproximately 200 g were used. They were obtained from Orion Ltd, Finland. The animals were divided into 4 groups: group 1, controls, receiving saline injection, group 2, receiving a single intravenous cerium injection of 2 mg/kg of body weight as the chloride in physiological saline solution of pH 3.5–4.0, group 3, given sodium phenobarbital 0.5 mg/ml in drinking water for 10 days and group 4 receiving the same mount of cerium as group 2 after phenobarbital administered in the same way as in group 3. All the animals were decapitated 3 days after the injection of cerium. The livers were removed and rinsed with ice-cold 0.1 M Tris-HCl buffer containing of 1.15 % KCl, pH 7.4. The 20 % liver homogenates were prepared in the same buffer with a motor driven Potter-Elvehjem type homogenizer Microsomes were obtained by first centrifuging the homogenates for 20 min. at 15,000 × g and the supernatants for a further 1 hour at 105,000 × g. The microsomal pellet was resuspended in the same buffer so that 2.5 ml of suspension corresponded to 1 g of liver tissue. For the *in vitro* de termination of drug metabolism the incubation was carried out at 37 in a Dubnoff type of shaking incubator in an atmosphere of air The amounts of cofactors added were: Nicotinamide dinucleotide phosphate (NADP), 1.5 µmol glucose-6-phosphate, 50 µmol glucose-6-phosphate dehydrogenase (Fluka), 68 EU Tris-HCl buffer 0.1 M containing of 1.15 % KCl pH 7 4 to final volume usually of 5 ml. The enzyme pathways studied, the amount of substrat in µmol, the amount of microsomes as corresponding liver tissue in grams, the final volume, incubation time and the method references were as follows: Hexobarbital (enhexymalum NFN) oxidase (HBO) activity 2 µmol hexobarbital, 1 g of liver tissue, in 5 ml, 15 min., according to COOPER & BRODIE (1955)· Uridine diphospho glucuronyl transferase (UDPGT) activity 2.1 µmol of *p*-nitrophenol, 0.2 g of liver tissue, in 1.5 ml, 20 min., according to HENDERSON (1970)· benzpyrene hydroxylase (BPH) activity 50 µg of 3,4-benzpyrene, 0.1 g of liver tissue in 5 ml, 15 min., according to KUNTZMAN *et al.* (1966).

Cytochrome P-450 was determined as described by OMURA & SATO (1964). Glucose-6-phosphate dehydrogenase (G-6-PDHG) was determined from the 105,000 × g super natant according t LOHR & WALLER (1965). Blood glucose was measured by the glucose oxidase method (Boehringer) and the plasma free fatty acids (FFA) were titrated by the method described by TROUT *et al.* (1960).

Results

Cerium treated animals behaved normally and there were no differences in the food and water consumption as compared with the controls. The phenobarbital treatment increased the mean liver wet weight by about 23 0 / in both groups 3 and 4

Table I

Effect of cerium on some enzymes involved in drug metabolism in normal and phenobarbital treated rats.

Group	Treatment	Number of animals	HBO: µmol. hexob. metabolized/ 1hr/g liver	HBO: % of control	UDPGT: µmol. PNPGA formed/ 1hr/g liver	UDPGT: % of control	BPH: relat. fl. units × 10^4/ 1hr/g liver	BPH: % of control
1	Control	6	2.00 ± 0.12	100.0	1.83 ± 0.90	100.0	5.24 ± 1.44	100.0
2	Ce-treat.	6	0.68 ± 0.32	34.0	4.03 ± 2.50	220.2	1.80 ± 0.71	34.3
3	Phe-treat.	6	3.82 ± 0.20	191.0	10.77 ± 0.81	588.5	7.09 ± 2.36	135.3
4	Phe-treat. and Ce-treat.	6	3.09 ± 0.24	154.5	11.97 ± 2.20	654.1	5.35 ± 0.91	102.1

The enzyme activities are expressed as mean values ± S.D.

Table 2

Effect of cerium on Cyt P-450 and G-6-P DHG in normal and phenobarbital pretreated rats.

Group	Treatment	Number of animals	Cyt P-450		G-6-P DHG	
			O.D.-cm/g liver	% of control	units/g liver	% of control
1	Control	6	0.35 ± 0.10	100.0	3.35 ± 0.48	100.0
2	Ce-treat.	6	0.14 ± 0.09	40.0	6.35 ± 1.67	189.6
3	Phe-pretreat.	6	0.73 ± 0.20	208.6	8.68 ± 1.70	259.1
4	Phe-pretreat. and Ce-treat.	6	0.49 ± 0.20	140.0	7.82 ± 0.79	233.4

The activities are expressed as mean values ± S.D.

Effect of cerium and phenobarbital pretreatment on enzyme activity

The activities of hexobarbital, p-nitrophenol and 3,4-benzpyrene metabolizing enzymes in different groups are shown in table 1 and the liver content of cytochrome P-450 and the activity of G-6-PDHG in table 2. It can be seen that in all the parameters studied phenobarbital pretreatment almost entirely abolished the effects of cerium. This was also true when cerium caused an increase in the activity of UDPGT. As in the case of glucuronide formation, cerium also had an activating effect on G-6-PDHG from the soluble fraction of the liver. Even here the normalizing effect of phenobarbital pretreatment could be seen.

Table 3

Effect of cerium on blood glucose and plasma FFA in normal and phenobarbital pretreated rats.

Group	Treatment	Number of animals	Blood glucose mg/100 ml	% of control	Plasma FFA µeq./ml	% of control
1	Control	6	116.7 ± 8.1	100.0	0.290 ± 0.062	100.0
2	Ce-treat.	6	80.9 ± 9.2	69.3	0.610 ± 0.417	210.3
3	Phe-pretreat.	6	100.6 ± 17.9	86.2	0.370 ± 0.112	127.6
4	Phe-pretreat. and Ce-treat.	6	105.4 ± 17.9	90.3	0.359 ± 0.151	123.8

The concentrations are expressed as mean values ± S.D.

Changes in blood glucose and plasma free fatty acid levels

The changes in blood glucose and plasma FFA levels are listed in table 3. In accordance with our earlier findings (ARVELA & KÄRKI 1971) we again found that the blood glucose in cerium treated animals was significantly decreased and that the plasma FFA level was doubled as compared with the controls. Phenobarbital pretreatment alone did not have any significant effects on those parameters but in the phenobarbital pretreated group the effect of cerium was abolished.

Discussion

Our study shows that phenobarbital pretreatment had a clear protective effect against cerium induced impairment of oxidative drug metabolism in rats. Phenobarbital pretreatment also almost completely abolished some enhanced enzyme activities caused by a single cerium injection. HOCHSTRATE & OBERDISSE (1970) have demonstrated that pretreatment with phenobarbital has a protective effect against liver injury caused by various hepatotoxic agents including praseodym. In experiments with rats they were able to show that the elevation of serum levels of GPT, GOT, LDH and SDH were prevented by phenobarbital pretreatment.

The most interesting effects are the cerium induced enhancements of UDPGT and G-6-P DHG activities. It is known that the UDPGT activity is increased after treatment with EDTA (HÄNNINEN 1968), sodium deoxycholate (VAN ROY & HEIRWEGH 1968), Triton X 100 (HEIRWEGH & NEUWESSEN 1968), digitonin (WINSNES 1969) and ultrasound (HENDERSON 1970). HÄNNINEN & PUUKKA (1970) have suggested that the active site of the enzyme in liver microsomes is surrounded by a lipid barrier and that the activation is caused by disruption of this barrier. Our study supports this assumption since it is possible that cerium which is known to react especially with phospholipids, similarly causes an unmasking effect on the active site. In order to explain the effect of phenobarbital treatment we suggest that in our study this inductive treatment, which is known to increase the phospholipide content in liver microsomes, causes a strenghtening of the orginal barrier.

Earlier investigations have shown that various hepatotoxins cause an increased activity of G-6-P DHG (PLATT & COCKRILL 1969). It has been suggested that changes in the activity of G-6-P DHG may be associated with the changes in glycogen metabolism (PLATT & COCKRILL 1969). The fact that cerium causes a depletion in the glycogen contents of the liver (MAGNUSSON 1963) supports this view. On the other hand the regenerative processes in the liver increase the need for reduced NADP and the fact that G-6-P DHG is the main producer of $NADPH_2$ could partly explain the increase in the activity of this dehydrogenase.

Cerium is known to reduce the oxidation of free fatty acids in the liver (SNYDER *et al.* 1960) and hence may cause their elevation in plasma. It is also possible that other organs, e.g., adrenal glands, may be affected by cerium and through increased catecholamine release play a part in the changes observed in lipid and carbohydrate metabolisms. We have found a 24 / decrease in the catecholamine content of the adrenal glands 2 days after cerium injection (unpublished results).

The mechanism by which cerium impairs drug metabolism is not clear The endoplasmic reticulum is an extremely delicate sheet made from proteins and phospholipids which holds associated enzymes in certain configurations. In contrast to UDPGT believed to be located in the rough part of endoplasmic reticulum, most drug metabolizing activity is found in the smooth part (FOUTS & GRAM 1969), and therefore may differ in sensitivity to the effect of membrane disruption caused by cerium.

Since the function of liver microsomes reflects changes in the original structure of the endoplasmic reticulum, our study seems to indicate that phenobarbital pretreatment results in a protective effect against cerium induced alterations of this structure.

Acknowledgements

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From the Department of Pharmacology University of Helsinki,
Siltavuorenpenger 10, 00170 Helsinki 17 Finland

Amphetamine Toxicity in Adult and Developing Mice

By

Eero Alhava

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Abstract: The dose-mortality curves for dl-amphetamine after intraperitoneal administration of 10–180 mg/kg of it to adult and developing mice in 7 age groups from 3 to 30 days were determined. The dose-mortality curve of adult mice was polyphasic. A 100 % mortality was obtained with 100 mg/kg of amphetamine. In developing mice the curves, polyphasic as well, were shifted to the right. At the age of 3 days the mortality caused by 180 mg/kg was only 68 %. With increasing age the animals became more sensitive to the drug, responding partially like the adults from the age of 18 days. The toxicity calculated in relation to the surface area remained unchanged, although the differences between the age groups of developing mice became smaller. In adult mice, pretreatment with 5 mg/kg of amphetamine protected against subsequent toxic doses of the drug, especially in the lower dosage ranges. Pretreatment given 4 hours before the toxic doses afforded the best protection. It is assumed that the immature CNS of developing mice is partially responsible for the increased tolerance to the drug. The polyphasic shape of the graphs and the protection afforded by the pretreatment reflect the complex lethal actions of amphetamine.

Key-words: Age – amphetamine – toxicity

Amphetamine has been used to improve the behaviour of hyperkinetic children for several decades (BRADLEY 1937 & 1950). This paradoxical tranquillizing effect is most pronounced in cases in which an organic lesion of the central nervous system (CNS) has been demonstrated. With increasing age the hyperkinetic syndrome usually disappears, and it has been suggested that delayed maturation of the CNS could be a factor influencing the favorable effect of amphetamine (MILLICHAP & FOWLER 1967). Although hyperkinetic children do not constitute a homogenous group, the paradoxical response and the increased tolerance to amphetamine indicate that age might be a factor influencing some effects of this drug.

Toxicity studies have constituted an important part of research on amphetamine. Amphetamine toxicity in aggregated and isolated mice has been

widely investigated by several investigators (MOORE 1963 CHERNOV et al. 1966) The mechanism of death caused by amphetamine is still unexplained. As reported by GARDOCKI et al. (1966a & b) the dose-mortality curve of dl-amphetamine is polyphasic, which might reflect the existence of several different modes of action, the structure of which has so far not been elucidated.

The purpose of this study was to analyze the role of age in amphetamine toxicity in mice.

Material and Methods

Experiments on adult mice

Adult male albino mice of NMRI strain weighing 24.6 ± 0.5 g were kept on the standard laboratory diet in their home cages (20 × 35 × 15 cm, 30–40 mice in each) until used. Dl-amphetamine sulphate dissolved in distilled water was injected intraperitoneally at 10 a.m., in a constant volume of 0.1 ml/10 g of body weight. The doses of amphetamine were calculated as base. After the injection the mice were put in groups of 4 animals into opaque plastic cages (15 × 20 × 13 cm) and observed for 4 hours. No food or water was given to the animals during the observation period. *Any mice which died in the 4 hours period were substituted by untreated animals in* order to maintain the grouped situation. Any additional deaths during the following 20 hours were noted.

To investigate the acute tolerance to amphetamine, male mice in groups of 4 were pretreated with dl-amphetamine 5 mg/kg intraperitoneally at various time intervals before the toxicity experiment was started No food or water was allowed during the observation period which lasted until 4 hours after the second injection. In order to eliminate the effect of fasting mice injected with saline served as controls. Any additional deaths up to 24 hours from the first injection were recorded.

Experiments on developing mice

Developing NMRI strain mice of either sex were used in the following age groups: 3, 6, 10, 14 18, 25 and 30 days. The body weights in the age groups were as follows: 3 days: 2.24 ± 0.13 g; 6 days: 3.29 ± 0.12 g; 10 days: 5.02 ± 0.22 g; 14 days: 5.55 ± 0.28 g; 18 days: 6.33 ± 0.24 25 days: 10.06 ± 0.47 g, and 30 days: 14.08 ± 0.44 g. The litters were kept with their mothers until the time of the experiments.

Dl-amphetamine was injected intraperitoneally in aqueous solution in a constant volume of 0.1 ml/10 g of body weight. In animals weighing less than 10 g the drug was injected by means of the Hamilton 100 µl microsyringe, the skin being pinched at the site of the injection in order to prevent leakage of the solution. During the observation period of 4 hours the mice were kept in groups of 4 animals, in similar environmental conditions as the adult mice. The groups were completed with untreated animals of the same age. Developing mice of the same age groups injected with saline served as controls. After the observation period the surviving animals were returned to the mothers. Any additional deaths occurring during the following 20 hours were counted. None of the dose mortality curves was completely achieved during a single day because of lack of litters. The experiments on the younger age groups (3, 6, 10 and 14 days) were completed in 8 weeks. The experiments on the 3 older age groups were completed during the subsequent 6 weeks. The environmental temperature was 24.5 ± 0.5 C.

Calculation of the results.

The mortality was expressed as a percentage, and the mean values and their standard errors (S.E.M.) were calculated. 1 20 mice represent each point of the dose-mortality curves. Student's *t*-test was used in comparing two means.

The dosage of dl-amphetamine given in mg/kg was reestimated in mg/m² in adult mice and in developing mice at the age of 3 days and 30 days, respectively. To convert a dose in mg/kg to a dose in mg/m² the approximate formula given by FREIREICH *et al.* (1966) was used: (dose in mg/m²) = (km) × (dose in mg/kg). The conversion factors (km) for each age group were obtained according to FREIREICH *et al.* (1966) by dividing the body weight (kg) by the surface area (m²), while the surface area was estimated as (kg)$^{2/3}$. The respective conversion factors (km) used were as follows. At the age of 3 days: km = 1.3, 30 days: km = 2.4 and adult mice: km = 2.9

Results

Adult mice.

The dose-mortality curves for amphetamine expressed on the mg/kg basis in grouped adult and developing mice are shown in figs. 1–2.

It can be seen from the figures that the mortality-response curve for amphetamine was polyphasic. According to GARDOCKI *et al.* (1966a) the classical

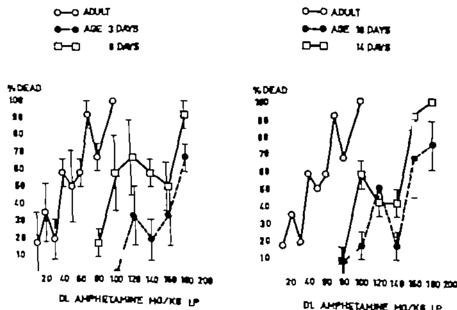


Fig. 1. The dose-mortality curves for dl-amphetamine in adult mice and younger age groups of developing mice. Each point of the curves

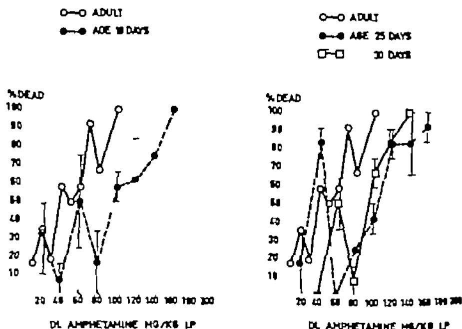


Fig. 2. The dose-mortality curves for dl-amphetamine in older age groups of developing mice as compared to that of adult mice.

LD_{50} expression is not applicable to this type of curve. Even though the curve cannot be definitely divided into ascending and descending segments, it is obvious that some of the higher doses of amphetamine were less toxic than lower doses. Since the dosage of amphetamine was less than 60 mg/kg the mortality did not exceed 60 / The gross effects observed following administration of amphetamine were qualitatively similar in the dosage range of 10–60 mg/kg. These were increased motor activity pilo-erection, sweating, Straub tail, tremor vocalization, sensitivity to touch, aggression and stereotypical movements. *The untreated mice used in completing the groups* also exhibited slight motor stimulation. Death seemed to result from exhaustion and respiratory depression. The majority of deaths occurring in this dosage range was recorded towards the end of the observation period. The time mortality relationships for most of the doses are presented in fig. 3

Doses higher than 60 mg/kg caused ataxia and marked convulsions. In this higher dosage range the mice died sooner most of deaths occurring within 2 hours after the injection. A 100 / mortality was obtained with 100 mg/kg of amphetamine, after which dose 82 / of the animals died in 30 minutes and the remainder in 60 minutes (fig. 3). No sedative effects could be observed with any dose of ampl

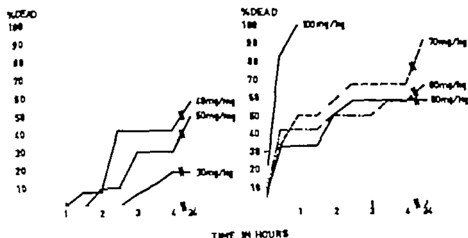


Fig. 3. Time-mortality relationships with various doses of amphetamine in adult mice.

Developing mice.

In developing mice of 7 age groups the toxicity curves were shifted to the right, indicating that young mice tolerated higher doses of amphetamine than adults. The effect of fasting, weaning and the injection *per se* was eliminated by the saline-injected controls, all of which survived.

At the age of 3 days all the animals survived after the administration of 100 mg/kg of amphetamine (fig. 1) Only a 68 / mortality was obtained with a dose as high as 180 mg/kg. With increasing age the animals became more sensitive to the drug, the graphs shifting gradually towards that measured in adults (fig. 1–2). The shape of the dose-mortality curves in all age groups studied was biphasic. In younger age groups (3 to 10 days) the de scending part of the curve was in the dosage range of 120–140 mg/kg (fig. 1). At the age of 14 days the descending part of the first peak was slightly shifted to the left, being in the dosage range of 100–140 mg/kg (fig. 1).

In the younger age groups (3 to 14 days) the behavioural effects caused by amphetamine were slight. Increased motor activity was seen in 14-day-old mice. Most of the deaths occurred within 30 to 60 minutes after the injec tion. The time-mortality relationships in 3 age groups of developing mice are presented in figs. 4–6.

The toxicity of amphetamine was increased from the age of 18 days onwards, the animals responding partially like the adults (fig. 2) In the older age groups (18 to 30 days) amphetamine caused increased motor ac tivity and the other behavioural effects described for adult mice. The highest

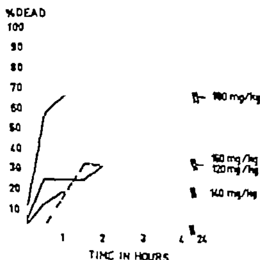


Fig. 4 Time-mortality relationships with various doses of amphetamine in mice aged 3 days.

doses caused ataxia and marked convulsions. The deaths caused by lower doses occurred mostly during the latter period of the observation time, while doses exceeding 100 mg/kg caused convulsions and sudden deaths within 30

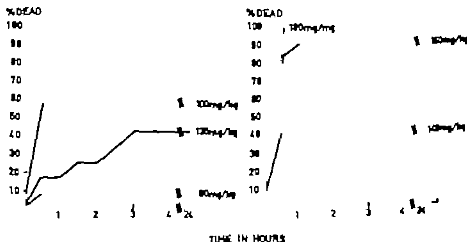


Fig. 5 Time-mortality relationships with various doses of amphetamine in mice aged 14 days.

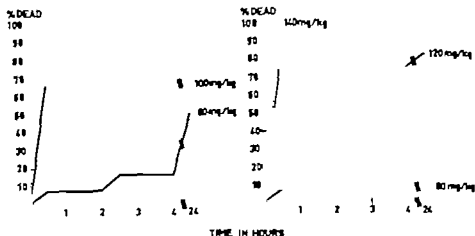


Fig. 6. Time-mortality relationships with various doses of amphetamine in mice aged 30 days.

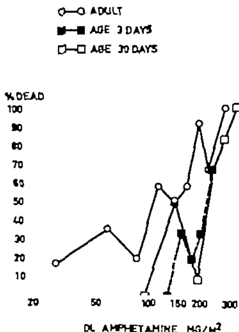


Fig. 7 Toxicity of amphetamine in adult and developing mice calculated on the basis of mg/m². The dosage is expressed in logarithmic scale.

minutes (figs. 5–6) No dose of amphetamine was sedative in any age group studied.

Toxicity calculated to the surface area.

The dose-mortality curves of amphetamine on the basis of mg/m² are presented in fig. 7 The per-square-meter dosage system shifted the curves of developing mice closer to the adult curve, but the main differences in amphetamine toxicity in adult and developing mice still remained. This method of dosage calculation supported the primary finding that developing mice are less susceptible to the lethal action of amphetamine than adult mice in most sections of the dosage scale. However the differences between the age groups of developing mice became less pronounced.

Effect of amphetamine pretreatment

Adult mice were pretreated with 5 mg/kg of amphetamine intraperitoneally 1 hour to 5 hours before the injection of toxic doses of the drug. The pretreatment dose caused a moderate stimulation but no deaths. The subsequent large doses of amphetamine were less toxic than without pretreatment, provided the interval was 2 hours or more (figs. 8–10) Pretreatment given 4

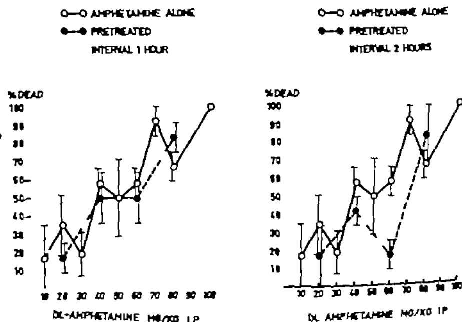
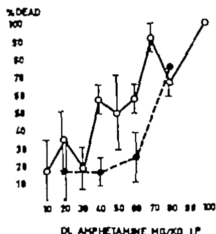


Fig. 8. Effect of amphetamine pretreatment (5 mg/kg) on its toxicity in adult mice. The pretreated animals were re-injected with toxic doses of amphetamine at various time intervals after the pretreatment. The curve of the pretreated mice is compared with that of mice given single injection of amphetamine.



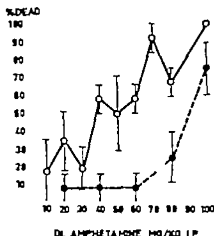


Fig. 9 Effect of amphetamine pretreatment (5 mg/kg) on its toxicity in adult mice. Toxicity experiments were performed 3 to 4 hours after the pretreatment.

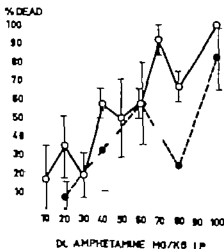


Fig. 10. Effect of amphetamine pretreatment (5 mg/kg) on its toxicity in adult mice. Toxic doses were injected 5 hours after the pretreatment.

hours before the toxic doses gave the best protection. Prolongation of the interval between the injections changed the shape of the dose-mortality curve. The gross behavioural effects due to toxic doses of amphetamine (sweating, licking, occasional bursts of running, stereotypical movements) were milder than those observed without pretreatment. Most of these effects were over in 1 hour

Discussion

The polyphasic dose mortality curve of amphetamine in adult mice is in accordance with the finding of GARDOCKI *et al.* (1966a & b) and reflects more than one intrinsic lethal actions of the drug. The two different patterns of behaviour preceding death by lower and higher doses of amphetamine in relation to the time of death also indicate the complex mechanisms of death. Whether these mechanisms are central or peripheral cannot be stated. It would appear that the central action of amphetamine is of primary importance with higher doses since death was preceded by convulsions (MOORE 1963).

The polyphasic dose-mortality curve could be reproduced in all age groups of developing mice. This also indicates two or more mechanisms of death in developing mice although these lethal actions appeared with higher doses of amphetamine.

It is widely accepted that the central action of amphetamine is dependent on the presence of catecholamines (CA) in the brain (WEISSMAN *et al.* 1966 HANSON 1967). The exact mechanism of the CA-releasing action is not known The behavioural excitation by lower doses of amphetamine is prevented by the blockade of noradrenaline synthesis by methyltyrosine (WEISSMAN *et al.* 1966 HANSON 1967) As to the amphetamine toxicity CA are also considered to be mediators (MOORE 1963) Large doses of amphetamine cause a marked release of the CA evidenced by the CA depletion in the mouse brain which causes strong behavioural excitation leading to exhaustion and these might be considered as important *lethal factors* (SMITH 1965 CARLSSON 1970)

A peripheral mechanism possibly contributing to the amphetamine toxicity is a blockade of neuromuscular transmission by high concentration of amphetamine (PETERSON *et al* 1964) This can be of importance as indicated by the breathing difficulties observed during convulsions at the high-dose level.

Developmental changes in monoamines in the mouse brain have been reported by AGRAWAL *et al.* (1968), the amount of the brain CA increasing with age. Thus, the biochemically and functionally immature CNS might be a factor influencing the low toxicity of amphetamine in developing mice,

particularly considering the necessity of an undisturbed CA-biosynthesis for the central action of amphetamine. Preliminary results from this laboratory give further support to the importance of the lack of brain CA in amphetamine toxicity in developing mice (ALHAVA, unpublished results 1971). Age dependent differences in the sensitivity of drug receptors may also be involved. Furthermore, BRIES & HERMAN (1971) found that adrenaline was 3 times and noradrenaline twice as toxic for adult as for neonatal mice. Hence, the immature nature of the peripheral noradrenergic system cannot be excluded as a factor involved in the low toxicity of amphetamine in developing mice.

Hyperthermia induced by amphetamine has been suggested as a fatal factor in amphetamine intoxication (MOORE 1963 1964). In grouped mice, reserpine produced a blockade of the hyperthermic response and a complete protection against the toxic effect of amphetamine, while methyltyrosine, with no action on body temperature, also protected against amphetamine toxicity (MENON & DANDIYA 1967). Furthermore, there was a lack of correlation between the effects of α and β-adrenergic blocking agents in the amphetamine group toxicity and hyperthermia in mice, indicating that hyperthermia may not be a primary factor in amphetamine toxicity (RAEVSKII & GURA 1970). In rabbits and rats the amphetamine induced hyperthermia and central excitation could be inhibited by the blockade of dopamine receptors (HILL & HORITA 1971 MATSUMOTO & GRIFFIN 1971). This gives further support to the importance of the immature CNS of developing animals, since brain dopamine reaches the adult level more slowly than the other monoamines (AGRAWAL *et al.* 1968) It is suggested that the immature adrenergic system may be a limiting factor in the development of hyperthermia and hence diminishes the exhaustion component of toxicity

Relevant studies on the influence of immaturity on drug absorption from various administration sites are not available. It could be assumed, however that amphetamine in aqueous solution administered intraperitoneally would be equally absorbed in adult and developing mice. Furthermore, differences in the distribution of amphetamine may play a significant role in the altered response of developing mice. The developmental state of the blood-brain-barrier may be a limiting factor in the uptake of amphetamine by the brain. GARATTINI (1969) has already reported that new-born rats have lower and more rapidly decreasing level of brain amphetamine than old rats. Preliminary results from this laboratory have shown that in young mice the brain amphetamine levels are lower and reach their peak very slowly (ALHAVA, unpublished results 1971)

The major metabolic pathways of amphetamine in mice are reported to be, firstly an aromatic hydroxylation to *p*-hydroxyamphetamine which may be excreted partly unchanged and conjugated with glucuronic acid and,

secondly an oxidative deamination to the ketone (BENAKIS & THOMASSET 1970). Although the toxicity of amphetamine and its metabolites has not been reported in the immature organism, it is unlikely that differences in the amphetamine metabolism between adult and developing mice could explain the increased tolerance to the drug in the young age groups.

PINKEL (1958) pointed out that the usual doses of certain drugs in various animal species and man were comparable when the dose was determined on the basis of mg/m² of surface area. Done (1964) believes that the real rationale of "the surface area rule" probably lies in the fact that the extracellular fluid volume is relatively constant on a surface area basis throughout life. In studies on the toxicological properties of amphetamine the most commonly used basis of dosage calculation is mg/kg. In this study therefore, it seemed to be reasonable to re-analyze the doses of amphetamine as based also on units of body surface area. This manner of dosage calculation did not definitely alter the original finding, although the difference between age groups of developing mice became smaller In this study the calculation of dosage in mg/kg seems to be adequate, since no comparisons within different species with variable conversion factors were made

The protective effect of amphetamine pretreatment against its own toxicity was maximal when the time interval beween the two injections was 4 hours. At this time interval the original polyphasic shape of the mortality response curve too had disappeared, appearing again when the interval was prolonged to 5 hours. This time-dependent protection of amphetamine pretreatment against its toxicity especially in the lower dose range could again reflect several lethal mechanisms of amphetamine, of which one at least is eliminated by the pretreatment. It is tempting to assume that this pretreatment-sensitive mechanism is mediated by CA since pretreatment caused a characteristic CA-dependent excitation (MOORE 1963 SMITH 1965). Whether the desensitization to the amphetamine toxicity is due to inadequate levels of central CA or changes in the receptor sensitivity is at present not known.

In conclusion amphetamine toxicity in mice was dependent on the age of the animals, the susceptibility to amphetamine increasing with age. This was assumed to be due to the biochemically and functionally immature state of the CNS of developing mice, and differences in the pharmacokinetics of amphetamine. The protective action of amphetamine pretreatment against its own toxicity most clearly found with lower doses of amphetamine may be attributable to an elimination of the CA-dependent component of toxicity

Acknowledgements

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From the Department of Pharmacology University of Helsinki,
Helsinki, Finland

Age and Brain Catecholamine Content as Factors *Influencing Amphetamine Toxicity in Mice*

By

Eeva Alhava and Erik Klinge

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Abstract. The brain noradrenaline (NA) and dopamine (DA) content of adult and developing mice of 3 age groups (3–5 13–15 and 32 35 days) was assayed and found to increase with age. In adult mice 4 hrs after the administration of 50 mg/kg of amphetamine the brain NA content was decreased by 65 %. After 100 mg/kg of amphetamine the mice died in 25 min., but the NA and DA levels were only slightly decreased. In all age groups of developing mice, the doses of amphetamine sufficient to be lethal in 30 min. (100–160 mg/kg) caused only slight decrease or even an increase in brain NA and DA levels. In developing mice surviving 60 min. after amphetamine, the brain amines were massively depleted. In adult mice the brain NA content in 1 hr and 4 hrs after the pretreatment dose of 5 mg/kg of amphetamine decreased by 20 %. The dose of 60 mg/kg 4 hrs after the pretreatment caused massive depletion in brain NA and DA content 1 hr after the latter injection. Even though the mice survived. The lack of correlation between brain catecholamine depletion and amphetamine toxicity reflects the complex lethal mechanisms induced by the drug.

Key-words: Age amphetamine – catecholamines.

In a previous study (ALHAVA 1972) the toxicity of amphetamine in mice was found to be dependent on the age of the animals, the susceptibility to amphetamine increasing with age. The polyphasic dose-mortality curve of amphetamine originally reported by GARDOCKI *et al.* (1966) and GEORGE & WOLF (1966) in adult mice, could be reproduced in all age groups of developing mice. It was assumed, that there are complex lethal mechanisms of amphetamine both in adult and developing mice.

There is general agreement as to the importance of the presence of catecholamines (CA) in the brain for the central action of amphetamine (WEISSMAN *et al.* 1966 HANSON 1967) In amphetamine toxicity the massive CA release caused by large doses of this amine is assumed to play a role in

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et al. (1958). Dopamine (DA) was assayed according to CARLSSON & WALDECK (1958). The whole brains were homogenized while cooled in 8.0 ml of 0.4 N perchloric acid. The adult brains were analyzed individually. In developing mice each sample consisted of 4 pooled brains. The amount of water in the brain tissue of adult mice was found to be 80 % and in the tissue of developing mice 85 % respectively. In the separation procedure Dowex 50-X4 resin (Fluka AG 200 to 400 mesh) was used in hydrogen form. The dimensions of the columns were 12.6 mm² × 52 mm. A detailed description of all the steps is given by KLEMOLA (1970). In 27 determinations the recovery of NA was 70.6 ± 2.4 % and that of DA 74.0 ± 2.2 %. The results were corrected for the respective recoveries.

The columns were loaded with pure CA plus dl-amphetamine to study the effect of amphetamine on the recoveries of CA. Dl-amphetamine did not interfere with the assay of NA and DA.

Doses of dl-amphetamine refer to the base. The environmental temperature during the experiments was 24.5 ± 0.5. The brain NA- and DA-values are expressed in µg/g of fresh tissue. Student's t-test was used for statistical analysis of the results.

Results

Age and brain CA content

The effect of age on the brain CA levels is presented in table 1. The brain NA and DA levels increased with age, almost reaching the mature level at the age of 32–35 days. At all stages of development the actual concentration of DA was higher than that of NA. The increase of the brain CA content was statistically significant in all age groups except from the age of 3–5 days to 13–15 days.

Table 1

Brain NA and DA content in developing an adult mice. Values are expressed in µg/g of fresh tissue ± S.E.M. Each experiment on developing mice was performed on 4 pooled brains. The adult mouse brains were analyzed individually

Age days	n	NA µg/g Mean±S.E.M.	P <	% of adult level	DA µg/g Mean±S.E.M.	P <	% of adult level
3–5	6	0.16 ± 0.01	0.001	37	0.18 ± 0.03	0.001	20
13–15	6	0.20 ± 0.02	0.001	47	0.24 ± 0.02	0.001	27
32–35	6	0.36 ± 0.01	0.01	84	0.66 ± 0.01	0.01	73
Adult	10	0.43 ± 0.01		100	0.90 ± 0.05		100

n = number of experiments.
The significances indicate deviations from the adult levels.

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Table 2

Effect of amphetamine on brain NA and DA levels in adult mice. Mice given 50 mg/kg of amphetamine were decapitated 4 hrs after the administration. The highly toxic dose of 100 mg/kg of amphetamine killed the animals within 15–25 min.

Dose of amphetamine mg/kg	n	Time after administration	NA µg/g Mean±S.E.M.	P <	% of control level	DA µg/g Mean±S.E.M.	P <	% of control level
50	8	4 hrs	0.15 ± 0.02	0.001	35	0.81 ± 0.06		90
100	5	15–25 min.	0.34 ± 0.01	0.01	79	0.64 ± 0.08	0.02	71
Controls	10		0.43 ± 0.01		100	0.90 ± 0.05		100

n = number of experiments.
The significances indicate deviations from the control levels.

Effect of amphetamine on brain CA content

Adult mice. The effect of two doses of amphetamine, 50 mg/kg and 100 mg/kg, on brain NA and DA levels was studied. The results are given in table 2. Four hours after the administration of 50 mg/kg of amphetamine the brain NA content was decreased by 65 / but the DA remained almost unchanged. After the administration of 100 mg/kg of amphetamine the mice had convulsions and died within 15–25 minutes. Both the NA and DA levels were slightly but significantly decreased.

Table 3

Effect of the pretreatment dose of 5 mg/kg of amphetamine on brain NA and DA levels in adult mice. The pretreatment dose was followed after 4 hrs by the administration of 60 mg/kg of amphetamine. The animals were decapitated 1 hr after the latter injection.

Dose of amphetamine mg/kg	n	Time after administration hrs	NA µg/g Mean±S.E.M.	P <	% of control level	DA µg/g Mean±S.E.M.	P <	% of control level
5	10	1	0.34 ± 0.02	0.01	79	0.78 ± 0.05		87
5	4	4	0.34 ± 0.01	0.01	79	0.87 ± 0.12		97
5+60	8	4+1	0.19 ± 0.01	0.001	44	0.36 ± 0.06	0.001	40
Controls	10		0.43 ± 0.01		100	0.90 ± 0.05		100

n = number of experiments.
The significances indicat deviations from the control levels.

The effect of the pretreatment dose of 5 mg/kg of amphetamine on brain NA and DA content is presented in table 3. The brain NA content decreased in 1 hour and in 4 hours after the pretreatment by about 20 %. The slight decrease in the DA content was insignificant. One hour after the pretreatment the mice showed typical stimulation, while the amphetamine-induced excitation was completely terminated 3 hours later. The dose of 60 mg/kg given 4 hours after the pretreatment caused a highly significant decrease ($P < 0.001$) in the CA content in the brains of mice decapitated 1 hour after the latter injection. The mice were not markedly stimulated at the time of decapitation.

Developing mice. The effect of various doses of amphetamine on brain NA and DA levels in the age group of 3–5 days is shown in table 4. In this youngest age group mice dying within 30 minutes showed no change in NA and DA content except a slight increase in NA (19 %) after 140 mg/kg of amphetamine ($P < 0.05$). In animals surviving 60 minutes, NA was depleted to 44 % of the control level by 120 mg/kg of amphetamine ($P < 0.01$). DA remained unchanged, or was increased by 78 % after 100 mg/kg of amphetamine ($P < 0.02$).

In the age group of 13–15 days the effect of amphetamine on brain CA content is given in table 5. Eighty mg/kg did not kill the animals and 60 minutes after the injection there was a decrease in both the NA and DA levels (NA decreased to 50 %, $P < 0.01$, DA to 58 %, $P < 0.05$). A hundred mg/kg of amphetamine did not change the NA and DA content in the brains

Table 4

Effect of amphetamine on brain NA and DA levels in developing mice. Age: 3–5 days. Brains from the mice dying within 30 min. were analyzed separately from those surviving for 60 min. Each experiment was performed on 4 pooled brains. The number of experiments is given in brackets.

Dose of amphetamine mg/kg	Time of death: Dead in 30 min. NA µg/g Mean ± S.E.M.	Dead in 30 min. DA µg/g Mean ± S.E.M.	Survived 60 min. NA µg/g Mean ± S.E.M.	Survived 60 min. DA µg/g Mean ± S.E.M.
100			0.13 ± 0.02	0.32 ± 0.04* (5)
120	0.21 ± 0.02	0.24 ± 0.04 (5)	0.07 ± 0.02*	0.18 ± 0.01 (3)
140	0.19 ± 0.01	0.20 ± 0.03 (5)	0.10 ± 0.01	0.18 ± 0.04 (7)
160	0.16 ± 0.01	0.22 ± 0.02 (5)		
Controls			0.16 ± 0.01	0.18 ± 0.03 (6)

$P < 0.05$ difference versus controls.
** $P < 0.01$.

Table 5

Effect of amphetamine on brain NA and DA levels in developing mice. Age: 13–15 days. Each experiment was performed on 4 pooled brains. The number of experiments is given in brackets.

Dose of amphetamine mg/kg	Time of death: Dead in 30 min. NA µg/g Mean ± S.E.M.	Dead in 30 min. DA µg/g Mean ± S.E.M.	Survived 60 min. NA µg/g Mean ± S.E.M.	Survived 60 min. DA µg/g Mean ± S.E.M.
80			0.10 ± 0.02**	0.14 ± 0.04 (5)
100	0.18 ± 0.03	0.20 ± 0 02 (3)	0.07 ± 0.02*	0.07 ± 0 01***(7)
140	0.14 ± 0.01	0.18 ± 0.06 (4)		
Controls			0.20 ± 0.02	0.24 ± 0.02 (9)

P < 0.05 difference versus controls.
P < 0.01
P < 0.001.

of mice dying within 30 minutes. In survivers this dose caused NA depletion to 35 / of the control level and DA depletion to 29 / respectively (P < 0 001). A dose of 140 mg/kg killed the animals within 30 minutes, and the NA content was moderately decreased (to 70 / P < 0.05) The slight decrease in the DA content was insignificant.

Table 6 shows the changes in brain CA levels induced by amphetamine in the age group of 32–35 days. In survivers injected with 60–80 mg/kg of amphetamine the NA content was decreased to 36 / (P < 0 001). The decrease in the DA level caused by 60 mg/kg was 30 / (P < 0.001) and that caused by 80 mg/kg 55 / (P < 0.001) respectively Mice dying within 30 minutes after 100 mg/kg of amphetamine showed a decrease of about 40 / in the NA level (P < 0 05) The DA content was insignificantly reduced. In mice surviving after this dose the NA decreased by 64 / (P < 0.001). DA was depleted to 65 / of the control level (P < 0 001).

Discussion

The toxicological properties of amphetamine appear to be complex. The mice with a "normal brain content of CA were more susceptible to the toxic effect of the drug than the developing animals with low content of brain CA. However the toxic effects of amphetamine were not directly correlated with the total brain content of either NA or DA. The pretreatment study with a low dose of amphetamine showed no correlation between the

total brain levels of the CA and amphetamine toxicity since this pretreatment gave protection against the lethal effect but no protection against the CA depleting effect of a subsequent high toxic dose of amphetamine.

The developmental pattern of the CA content in mouse brain is in agreement with the finding of AGRAWAL *et al* (1968) As reported by several authors (WEISSMAN *et al.* 1966 HANSON 1967), the amphetamine-induced excitation is blocked by inhibition of tyrosine hydroxylase, the rate-limiting step in CA biosynthesis, with methyltyrosine while, according to MENON & DANDIYA (1967) complete protection against amphetamine toxicity in mice is also offered by methyltyrosine. The rate of maturation of CA in mouse brain indicates the slow development of those biosynthetic enzyme systems which are responsible for the endogenous biosynthesis of CA (AGRAWAL *et al.* 1968) *It is concluded, therefore, that the age-dependent tolerance* to the toxic effects of amphetamine previously observed (ALHAVA 1972) is due, at least partially to the biochemically immature CNS. Furthermore, the developmental pattern of CA in mouse brain is compatible with the findings of KOBAYASHI *et al* (1963), who reported that neuro-histological studies of the mouse cortex revealed mature morphological development by the 15th–17th day and the spontaneous electrocorticogram exhibited adult patterns by the 17th day The toxicity of amphetamine was increased from the age of 18 days onwards, the animals responding partially like the adults (ALHAVA 1972). This gives further support to the importance of the immaturity of the CNS in increased amphetamine tolerance in developing mice.

Table 6

Effect of amphetamine on brain NA and DA levels in developing mice. Age: 32 35 days. Each experiment was performed on 4 pooled brains. The number of experiments is given in brackets.

Dose of *amphetamine* mg/kg	Time of death: *Dead in 30 min.* NA μg/g Mean ± S.E.M.	*Dead in 30 min.* DA μg/g Mean ± S.E.M.	*Survived 60 min.* NA μg/g Mean ± S.E.M.	*Survived 60 min.* DA μg/g Mean ± S.E.M.
60			0.13 ± 0.01 **	0 46 ± 0 03***(7)
80			0.13 ± 0.02*	0.30 ± 0.02***(7)
100	0.29 ± 0.03*	0.61 ± 0.02 (7)	0.13 ± 0.06***	0.43 ± 0.03* (3)
Controls			0 36 ± 0.01	0.66 ± 0 01 (6)

P < 0 05 difference versus controls.
** P < 0.001.

In adult mice 50 mg/kg and 100 mg/kg of amphetamine induced two different patterns of behaviour and times of death (ALHAVA 1972). The brain CA responses to these doses were also different (table 2). The brain NA was depleted to 35 % of the control level at 4 hours after 50 mg/kg of amphetamine but only to 79 % in quickly dying mice given 100 mg/kg, which indicates that the reduction of the brain NA level is not the cause of death. This is not surprising since the brain CA can be massively depleted by methyltyrosine without any high mortality rate (CORRODI & HANSON 1966). The changes in brain DA were less pronounced, only a decrease to 71 % after 100 mg/kg being seen. This is in agreement with the observations made by SMITH (1965), who reported an increase in brain DA with low doses of d-amphetamine while there was a significant DA decrease with high doses. However, the dissociation between the brain CA depletion and the toxic effects of amphetamine does not exclude the role of the central CA in amphetamine toxicity (MENON & DANDIYA 1967). The studies made by GLOWINSKI (1970) reveal the complexity of the mechanisms of action of amphetamine on various metabolic processes of central CA neurons. The present findings do not give any further information about the lethal mechanisms in amphetamine toxicity.

In developing mice, the primarily low levels of brain CA were assumed to give at least partial protection against amphetamine toxicity (vide supra). The brain CA were particularly depleted in the surviving animals, while in mice dying within 30 minutes the brain NA and DA levels were slightly decreased or even increased (table 4). The ability of amphetamine to block the re-uptake of NA into neurons results in an increase in the levels of free NA at the receptor sites, which may potentiate the physiological effects of the CA (GLOWINSKI *et al.* 1966) and thus be an important event leading to death. Furthermore, the increase in brain CA levels in quickly dying young mice could be attributable to the dopamine-β-hydroxylase and monoamine oxidase-inhibiting activity of high doses of amphetamine (MOORE 1964, GLOWINSKI *et al.* 1966, RUTLEDGE 1970). During the neonatal development of various species there appear to be deficiencies in CA-degrading enzymes (MIRKIN 1970). Variations in the turn-over rate of the central CA may thus be of importance in the CA-responses to amphetamine in developing mice.

Reserpine also counteracts the toxic effects of amphetamine (LASAGNA & McCANN 1957, BURN & HOBBS 1958, MOORE 1964, MENON & DANDIYA 1967). Pretreatment with a single dose of reserpine afforded significant protection against the toxic effects of d-amphetamine during the 4-hour measurement period in mice (STOLK & RECH 1968). The best protection was given by amphetamine pretreatment at 4-hour interval (Alhava 1972). The brain NA content at 1 hour and 4 hours after the pretreatment was equally de-

creased, although the excitation seen at 1 hour was over 3 hours later One hour after the 60 mg/kg dose of amphetamine the mice were still surviving, not markedly stimulated, but both NA and DA were found to be depleted (table 3). Thus, there is no correlation between the total brain levels of the CA and amphetamine toxicity A time-dependent reserpine-like protective mechanism could be, at least partially responsible for the enhanced tolerance to amphetamine after the pretreatment. The pretreatment-sensitive component of toxicity especially observed with low doses of amphetamine (ALHAVA 1972) could be dependent on peripheral mechanisms or on the differences in the turn-over rate of the CA at various intervals after the pretreatment.

The importance of time in the protection provided by amphetamine pre treatment suggests that the metabolism of amphetamine might influence this phenomenon. The major metabolite of amphetamine in mice is *p*-hydroxyamphetamine (BENAKIS & THOMASSET 1970). In rats, *p*-hydroxyamphet amine can be β-hydroxylated to *p*-hydroxynorephedrine in peripheral adrenergically innervated organs (KOPIN *et al.* 1965), and this agent is also found in the CNS after the administration of amphetamine (GROPPETTI & COSTA 1969 COSTA & GROPPETTI 1970 BRODIE *et al.* 1970· LEWANDER 1970) The role of metabolites in the protection provided by amphetamine pretreatment remains obscure and is probably worthy of further study

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From the Institute of Pharmacology University of Oslo, Blindern, Oslo 3, Norway

Potassium-Efflux and the Contractile Response to Sympathomimetic Amines in Circular Rabbit Fundus Muscle

By

J F W Haffner R.-L. Nesheim and J Setekleiv

(Received February 16 1972 Accepted March 22, 1972)

Abstract The mechanical effects and the alterations in ^{42}K-efflux produced by carbachol, noradrenaline, adrenaline, phenylephrine and isoprenaline have been studied in circular strips from the fundus of rabbit stomachs. Carbachol, *noradrenaline adrenaline and phenylephrine caused sustained contractions of* the preparations, and produced a temporary but statistically significant increase in K-efflux. A rough correlation was found between the magnitudes of these two effects. Isoprenaline reduced tone in strips which were contracting in response to carbachol, but had no effect on K-efflux. It is suggested that the increase in tone and K-efflux produced by noradrenaline, adrenaline, and phenylephrine is due to stimulation of adrenergic α-receptors. Since the alteration in K-efflux is only temporary it would appear that K-efflux is associated with the excitatory processes, but not with those which maintain the contraction.

Key-words: K-efflux – stomach muscl – sympathomimetic agents – carbachol.

In most gastrointestinal muscle preparations both α- and β-adrenergic receptors mediate inhibitory responses. Circular preparations from the fundus of rabbit stomachs, however generally contract when exposed to α-adrenergic agents (HAFFNER 1971 & 1972) The contraction is sustained it lasts as long as the preparations are exposed to the excitatory agent. Phasic alterations in tension, which dominate the motility pattern in other gastrointestinal muscle preparations, are rarely seen in rabbit fundus strips. These motility characteristics make the rabbit fundus strips more suitable for studies of ionic fluxes than other gastrointestinal preparations, in which the *evaluation* of drug effects is complicated by the fluctuations associated with phasic activity

It has been shown that excitatory responses to agents other than the catecholamines are accompanied by an increased permeability to several ions, and it has also been shown that the inhibitory effects mediated by adrenergic α-receptors are associated with a selective increase of potassium permeability (for ref. see SETEKLEIV 1970). The ionic mechanisms underlying the excitatory α-adrenergic responses, are, however not known.

The present study was undertaken in order to determine how the sympathomimetic amines adrenaline, noradrenaline, phenylephrine and isoprenaline affect the K-efflux in circular preparations from the fundus of rabbit stomachs, and whether the effect on K-efflux is correlated with the mechanical effects of the drugs. The effects of carbachol on muscular tension and K-efflux were also studied for comparison.

Material and Methods

Female rabbits weighing from two to four kg were stunned by blow on the occiput and bled by cutting the carotid arteries. The stomach was removed, and circular strips were cut from the anterior surface of the fundus above the level of the cardiac sphincter The strips comprised the full thickness of the muscular wall, but the mucosa was cut away The preparations were stored until the next day or the day after (18–44 hrs) at 4 in Krebs solution containing (mM): Na 136.9 K 5.9 Ca^{2+} 2.5 Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 133.6 and glucose 11.5 The solution had been bubbled with 95 % O_2 and 5 % CO_2 which gives it a pH of 7.4 (Cold storage was used in order to have uniform pre-investigation periods for the different strips; it was thought better to investigate all the strips after cold storage than some which were completely fresh and some which had been stored. It has previously been reported that the response to the catecholamines is qualitatively unaltered after 24 hrs cold storage (HAFFNER 1971), the same applies for 48 hrs storage (unpublished results)).

The experiments were started by incubating the preparation for one hour in an organ bath containing oxygenated Krebs solution t 38 The solution had the composition given above, but contained radioactive ^{42}K, which gave specific activity of approximately 6 µcl/ml. After being loaded with radioactivity the strip was mounted in constant flow apparatus (BRADING 1958) which was perfused at a rate of 2–3 ml/min. with non-radioactive Krebs solution of the same composition and temperature as the incubation fluid. The transfer and mounting took approximately 2 min. The preparation was fixed at one end, the other end was tied to an isometric Grass force-displacement transducer (Model FT03C) connected to Grass polygraph (Model 7W 12 PA). The preparations were stretched to an initial tension of one to two gram, and the muscular tension was recorded for the remainder of the experiment. The effluent solution containing ^{42}K from the strips was collected in plastic test-tubes which were changed every two minutes by an automatic fraction collector (LKB Ultrarac 7000). The radioactivity of each sample was determined in a Packard Autogamma Spectrometer Model 2001.

After an initial 32 min. equilibration and control period, the drugs were added to the perfusing solution, and the strips were perfused for further 32 min. period. In the experiments where the effect of isoprenaline 10^{-6}M in the presence of 10^{-7}M carbachol was tested, carbachol was added to the preparation after an equilibration period of only 20 min. and isoprenaline was added 20 min. later

After the experiments were completed, the radioactivity remaining in the preparation was determined, and the strips were dried t 60° for 48 hours before the dry weight was determined. Each concentration of drug was tested on 4–6 preparations. The efflux curves which were obtained were compared with the curves from 12 control preparations which had not been exposed to any drug.

The drugs used carbacholine chloride, phenylephrine HCl (metaoxedrinum NFN), adrenaline bitartrate, noradrenaline bitartrate and isoprenaline sulphate, were added to the perfusate in amounts which gave final concentrations ranging from 10^{-7}M to 10^{-4}M. The drugs were prepared each day from stock-solutions kept in the deep-freeze. Radioactive potassium (^{42}K) was supplied by Institutt for Atomenergi, Kjeller Norway

Calculations

The content of ^{42}K in the preparation throughout the experiment was calculated by adding the radioactivity of the samples of effluent to the radioactivity which remained in the preparation at the end of the experiment. The fraction lost at any time during the experiment was determined by dividing the amount of tracer lost per min. with the amount of tracer which the preparation contained at that time.

The effect of each drug was recorded as the maximal efflux of ^{42}K after addition of the drug. The results are expressed in per cent of the ^{42}K-efflux at the time when the drug was added.

Results

Contractile effects

Carbachol, adrenaline, noradrenaline and phenylephrine all caused an increase in tension of the preparations. The contractile response reached a maximum in 2 to 8 min. and always remained at this maximal level for the remainder of the experiment. The magnitude of the response varied with the

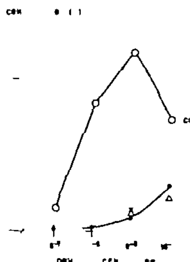


Fig. 1. Contractile force in circular rabbit fundus strips. Log dose-response curves for carbachol (CC) ○, adrenaline (A) ×, phenylephrine (PE) ● and noradrenaline (NA) △ *Abscissa*. concentration of drug in M. *Ordinate*: force in gram. *Each point* represents the mean of 4–6 preparations.

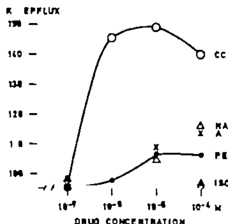


Fig. 2. ⁴²K-efflux in circular rabbit fundus strips. Log dose-response curves for carbachol (CC) ○, noradrenaline (NA) △, adrenaline (A) ×, phenylephrine (PE) ● and isoprenaline (ISO) ▲. *Abscissa*: concentration of drug in M. *Ordinate*: maximal efflux of ⁴²K produced by the drugs as per cent of efflux immediately before addition of drug. Each point represents the mean of 4–6 preparations.

drug and the concentration used, carbachol being by far the most potent drug (fig. 1). Isoprenaline had no effect unless it was given to strips which were contracted in response to carbachol, in which case it reduced the tension.

K-efflux

Typical fraction lost curves for potassium efflux were obtained. After an initial rapid drop, the curves flattened out, and after approximately 15 min. the fall was slow and linear probably reflecting outward transfer of ⁴²K across the cell membrane. The half-time for K-efflux for this part of the curve (calculated from the difference in efflux at 20 and 60 min.) in the controls was 64.5 min. In the control experiments the fall continued to be linear until the experiments were completed. When carbachol, noradrenaline, adrenaline or phenylephrine was added, a temporary increase in efflux occurred. The magnitude of this alteration varied with the drug used and also seemed to be dose-dependent for the individual drugs. Fig. 2 shows log dose-response curves for the increased K-efflux produced by carbachol and the catecholamines. As can be seen from the curves, carbachol is the most potent drug in this respect, increasing the K-efflux to 140–150 / in concentrations of 10^{-6} 10^{-5} and 10^{-4}M. Noradrenaline, adrenaline and phenylephrine are much less and approximately equally potent, increasing the K-efflux to 107–116 / (in the control curves a fall to 97.8 / was observed in the corresponding interval) The significance of the increase in efflux tested against

the controls was determined by the Wilcoxon two sample test. The increases produced by 10^{-4} 10^{-5} and 10^{-6} carbachol are *highly significant* ($P < 0.01$). The increases in response to the two highest concentrations of noradrenaline and phenylephrine, 10^{-4} and 10^{-5}M are also significant ($P < 0.05$), as is the increase in response to 10^{-4}M adrenaline ($P < 0.01$). Isoprenaline had no effect on K-efflux compared with the controls. As it was thought that this might be due to the lack of effect on the contractile state, some strips were contracted with carbachol 10^{-7} before isoprenaline 10^{-4} was added. No alteration occurred in the flux, although isoprenaline now induced a loss of tension.

The relationship between contractile force and K-efflux.

Carbachol and the three sympathomimetic amines which stimulate the α-adrenergic receptors increase both K-efflux and tension. However no direct correlation could be established between the two effects. Within each group of observations with one particular dose of a drug, there was no direct correlation between the relative magnitudes of the contractile responses and the corresponding increases in K-efflux. The time-course of the contractile responses and the increase in K-efflux also varied. As stated previously the contractile response reached a maximum in 2 to 8 min., and the tension remained at this high level for the remainder of the experiment. The K-efflux also reached a maximum in 2 to 8 min., but the increase was only temporary subsiding 10–20 min. after the addition of drug. Figs. 3 and 4 show alterations in tension and K-efflux throughout typical experiments with carbachol and

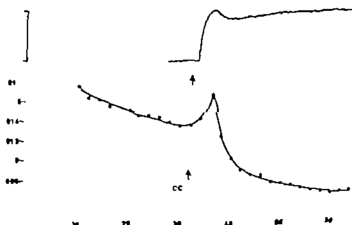


Fig. 3 Tension and ^{42}K fraction lost-curves from typical experiment. *Abscissa*: time in min. *Ordinate*: tension (upper tracing) and fraction lost (lower tracing). At the arrow carbachol (CC) 10^{-6}M was added. Carbachol causes a transient rise in ^{42}K-efflux and a sustained contraction.

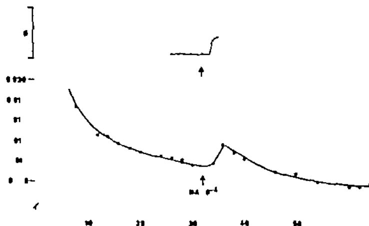


Fig. 4 Curves as fig. 3. At the arrow noradrenaline (NA) 10^{-4}M was added.

noradrenaline. Fig 5 shows that there is no relation between the inhibitory effect of isoprenaline and K-efflux. The figure also shows that the concentration of carbachol used (10^{-7}M), is insufficient to produce any definite alteration in K-efflux in this particular preparation, although the tension is increased, which indicates that the threshold for the drug-induced increase in ⁴²K-efflux is higher than the threshold for contraction.

Discussion

The present experiments show that the contractile effect of carbachol in circular rabbit fundus muscle is associated with an increase in K-efflux. Similar effects of cholinergic stimulation have previously been reported in other types of smooth muscle (Born & Bülbring 1956 Lembeck & Strobach 1956 Hurwitz 1960 Durbin & Jenkinson 1961 Weiss *et al.* 1961 Banerjee & Lewis 1964 Spero 1967 Burgen & Spero 1968).

⁴²K-efflux was also increased when the circular fundus strips were stimulated with the α-adrenergic receptor stimulating agents phenylephrine, adrenaline and noradrenaline. The relationship between K-efflux and the excitatory effect of α-adrenergic receptor stimulation has not been studied previously but it has been shown that the inhibitory effects of α-receptor stimulation are associated with increased K-efflux (Born & Bülbring 1956 Jenkinson & Morton 1965 Bülbring *et al.* 1966). Thus it would appear that α-adrenergic receptor stimulation always increases K-efflux, and that the quality of the mechanical response (inhibitory or excitatory) is determined by other factors.

Electrical recordings may shed some light on this. It is generally assumed

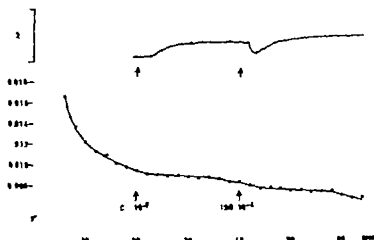


Fig. 5 Curves as fig. 3 At the arrows, carbachol (CC) 10^{-7}M and isoprenaline (ISO) 10^{-6}M were added. Carbachol 10^{-7}M causes a contraction, but no measurable rise in ^{42}K-efflux Isoprenaline 10^{-6}M causes a fall in tension, but no change in 42K-efflux. The fluctuations in the fraction lost curves in these experiments are not significantly different from those observed in the controls.

that the resting membrane potential is determined by the ionic membrane permeabilities, and as those appear to differ in different smooth muscle tissues, it is possible that an increase in K-permeability might affect different types of smooth muscle differently At present very little is known about the electrical activity during excitation of circular rabbit fundus muscle. FURCHGOTT (1960) has suggested that the concentration in response to acetylcholine coincides with membrane depolarization without action potentials, and that adrenaline contractions are accompanied by hyperpolarization. This seems unlikely since prolonged contractions of this type (often called "contracture") are associated with sustained depolarization in other smooth muscle preparations (see AXELSSON 1970). Moreover preliminary experiments in our laboratory show that the contractions produced by acetylcholine and potassium in rabbit fundus strips are associated with spike discharge and sustained depolarization. As yet we have not studied the relationship between electrical and mechanical response to α-adrenergic stimulation, but it seems likely that it should be the same as that of the other excitatory agents.

In the present investigation it was found that the time-course of the excitatory agents on ^{42}K-efflux and mechanical activity differed the alteration in efflux was temporary while the mechanical effect was sustained. The temporary nature of the alteration in ^{42}K-efflux and its rough correlation with the time required to reach maximal contractile effect, suggest that the increase in ^{42}K-efflux is associated with the excitation process, but not with the maintenance of contraction. It is unlikely that the increased efflux of

potassium should be due to extra- or intracellular mechanical squeezing, as a similar increase in K-efflux is obtained in preparations which give inhibitory responses to α-adrenergic stimulation (SETEKLEIV 1970). Moreover as isometric recording was used in the present study only minimal configurational alterations of the preparations were possible, and hence no extracellular squeezing could occur It should also be noted that small doses of carbachol increased the tension without affecting the ^{42}K-efflux (fig. 5) This is in accordance with the findings of BURGEN & SPERO (1968) in guinea-pig intestinal muscle.

The relative magnitudes of the alterations in K-efflux and the mechanical responses were roughly similar for the drugs used in the present study This is what one would expect if the alteration in ^{42}K-efflux reflects the extent of excitation. No direct relationship can be expected since the magnitude of the K-efflux mainly depends on the total mass (cell surface) of the preparations (thickness × length) whereas the magnitude of the mechanical response mainly depends on the thickness (cross-sectional area) and degree of stretch (passive tension per unit cross-sectional area).

In the present investigation, exposure to the β-adrenergic receptor stimulating agent isoprenaline had no effect on ^{42}K-efflux, not even when the drug produced relaxation. This is in accordance with the findings of other investigators (see SETEKLEIV 1970 for references). The inhibitory effects of the β-adrenergic agents appear to depend on intracellular metabolic alterations (BRODY & DIAMOND 1967 ANDERSON & MOHME LUNDHOLM 1970).

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From the Department of Alcohol Research, Karolinska Institutet,
S-104 01 Stockholm, Sweden

4-Methylpyrazole as an Inhibitor of Ethanol Metabolism Differential Metabolic and Central Nervous Effects

By

Ulf Rydberg and Aldo Neri

(Received February 16, 1972, Accepted April 13, 1972)

Abstract The properties of 4-methylpyrazole (4-MP) as an inhibitor of ethanol metabolism were investigated in rats. No effect was seen on enzymatic determination of ethanol. 4-MP in doses of 0.0053 – 3.0 mmol/kg brought about a competitive inhibition of the metabolism of 32.6 mmol ethanol per kg body weight ranging from 20.8 to 80.5 % A dose of 0.017 mmol 4-MP per kg gave inhibition of 50 % By varying the interval between the administration of 4-MP and ethanol from 15 min. to 48 hrs, it was found that the inhibitory effect disappeared after 12 hrs. The influence of 4-MP on ethanol-induced incoordination was measured by a modified tilting-plane technique. The mean maximal impairment was significant in all conditions, when compared to the controls. For ethanol alone the mean maximal impairment was 11.9 % for 4-MP alone 7.2 % and for ethanol + 4-MP 14.9 % the effect lasting for 8 hrs. Thus, 4-MP exerts significant central depressant effects *per se*, and produces an enhancement and prolongation of ethanol-induced effects on coordination in the rat.

Key-words. Alcohol ethyl/metabolism – alcoholic intoxication – drug antagonism – drug synergism – pyrazoles – rats.

The inhibitory effect of 4-methylpyrazole on ethanol metabolism *in vivo* in rats was first suggested by LESTER *et al.* (1968) In investigations by BLOMSTRAND & THEORELL (1970) and by BLOMSTRAND (1971) it was found that *the administration of 4-methylpyrazole resulted in a marked inhibition* of the elimination of ethanol.

In a number of experiments 4-methylpyrazole was also administered to human subjects (BLOMSTRAND & THEORELL 1970 BLOMSTRAND 1971) When given in a dose of 0 084 mmol/kg, 4-methylpyrazole decreased the recovery of expired $^{14}CO_2$ by about 50 / after the administration of [1-^{14}C]-ethanol as measured by radiorespirometry

The administration of pyrazole before ethanol gave an increased behavioural depression (GOLDBERG & RYDBERG 1969 BLUM *et al* 1971 RY-

1971) 3-methylpyrazole, a non inhibitor of alcohol dehydrogenase, also exerted similar effects when given before ethanol (BLUM *et al.* 1971). The central nervous effects of 4-methylpyrazole are not known.

The *aim* of the present study was (1) *to study* the dose-dependence of the interaction between 4-methylpyrazole and ethanol metabolism *in vivo* in rats, and (2) to elucidate whether 4-methylpyrazole has any central depressant effects or modifies ethanol-induced effects on the central nervous system, and also to correlate the degree of impairment to the concentration of ethanol in the blood.

Material and Methods

Material.

Male Sprague-Dawley rats with a mean initial weight of 199 g were used (n = 85). The animals had free access to tap water and pelleted diet (Anticimex Ltd.) until the start of the experiment.

Chemicals

Ethanol (Vin- & Spritcentralen Ltd.), 95 % w/v was diluted with 0.9 % saline to a concentration of 14.9 % w/v. Ethanol was injected intraperitoneally in a standard dose of 32.6 mmol/kg (1.5 g/kg).

4-Methylpyrazole as the hydrochloride (*M* = 118.5) was synthetised according to PINO & ERCOLI (1951) by Dr. B. Sjöberg, Astra Ltd., Södertälje. It was used in 0.1 or 2.0 % (w/v) solutions in saline, and was injected intraperitoneally.

Blood sampling

Blood samples, 10 µl, were withdrawn in duplicate from the tip of the tail at 30 and 60 min. after the administration of ethanol and then at 60 min. intervals up to 10 hrs. The samples were taken with dispensable pipettes (Microcaps, Drummond Ltd.) and were transferred into 2 ml Auto Analyzer cups with 1.0 ml sodium fluoride solution, 0.2 % w/v. Blood samples were also withdrawn in the control conditions.

Ethanol determination.

Enzymatic determinations were carried out either by the automated ADH dialysis method (GOLDBERG & RYDBERG 1966) or by the ultramicro destillation method (BURTEN, unpublished results). The S.E.M. of a duplicate sample was ± 0.019 mg ethanol per ml blood with a mean concentration of 0.83 mg/ml. To establish the possible influence of 4-methylpyrazole on the methods used for ethanol determination, blood samples from experiments in which rats were given ethanol together with 4-methylpyrazole were analyzed for ethanol by both methods.

Ethanol parameters.

For definitions of ethanol parameters see WIDMARK (1930).

Determination of changes in coordination.

The tilting-plane technique by ARVOLA *et al.* (1958) has been modified by GOLDBERG *et al.* (1972). The surface, made of hard board, rough side up, was surrounded by a transparent plastic wall and was curved in front, with a piece of foam rubber attached

at the bottom of the plane. After the rat was settled on the plane, head towards the curved front, the plane was pulled by a string connected to an electric motor and tilted at a constant speed from 0 to 90 degrees in 8 sec. When the animal started to slide, the tilting was stopped by switch, and the angle was recorded. In the present investigation, a further modification was introduced. When the animal started to slide on the tilting plane, it passed the beam of a photo cell. At that moment the motor pulling the plane was stopped, and the sliding angle was determined. The determination was repeated 3 times on each occasion. The drug effects were estimated by comparing the performance scores during each of the drug conditions to the scores in the control condition. In order to correct for any differences with regard to initial level, all the measurements were expressed in per cent of the base line value obtained in the control condition.

The change in degree of coordination as measured by the tilting-plane technique, was studied after the administration of 32.6 mmol ethanol per kg and/or 0.084 mmol 4-methylpyrazole per kg body weight. The results were related to the blood ethanol concentrations. The series comprised five conditions for each of three groups of 8 animals. (1) Pre-drug condition (saline + saline) (2) 4-Methylpyrazole + saline (3) Ethanol + saline (4) Ethanol + 4-Methylpyrazole (5) Post-drug condition (saline + saline). Conditions 2–4 were rotated in a random order according to a modified Latin Square procedure, one week apart.

The *control* group (8 animals) received only saline + saline once a week for five weeks.

The experiments started at 8 a.m. with two initial determinations. 30 min. later 4-methylpyrazole or saline was administered, and 15 min. later ethanol or saline was given. Tilting tests were then carried out at hourly intervals for 10 hrs.

Results

Effect of 4-methylpyrazole on methods for ethanol determination.

When rats were given 32.6 mmol ethanol per kg body weight and 4-methylpyrazole in doses up to 3.0 mmol/kg, no differences in the ethanol values could be detected ($P > 0.5$) when determinations were performed by the automated dialysis method or by the ultra-micro distillation method. Thus it could be concluded that 4-methylpyrazole in the concentrations used exerted no inhibitory influence on the yeast alcohol dehydrogenase utilized in the determination method. Also higher concentrations of 4-methylpyrazole added to the incubation mixtures *in vitro* (0.084 – 8.4 mM) had no inhibitory effect on YADH.

Effects of 4-methylpyrazole on ethanol metabolism.

1 Ethanol experiments The mean blood ethanol curve after the intraperitoneal administration of 32.6 mmol ethanol per kg body weight is shown in fig. 1 lower part (n = 24). The linear decline of the curve is evident. The mean time until blood ethanol concentration reached zero was 2.9 min. The mean rate of elimination was 8.0 µg ethanol per ml per min. Another

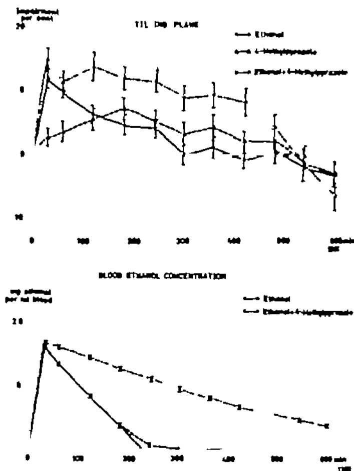


Fig. 1 *Upper part:* Change in degree of impairment with time after 4-methylpyrazol (0.084 mmol/kg) or ethanol (32.6 mmol/kg) and after 4-methylpyrazole (0.084 mmol/kg) + ethanol (32.6 mmol/kg) compared with the scores in the control condition. Means ± S.E.M. *Lower part:* Blood ethanol curves after ethanol (32.6 mmol/kg) and ethanol (32.6 mmol/kg) + 4-methylpyrazole (0.084 mmol/kg). Means ± S.E.M.

group of 8 animals gave similar results. Further data on blood ethanol parameters are given in table 1

2 *4-Methylpyrazole and ethanol.* The blood ethanol curve after the administration of 4-methylpyrazole is shown in fig. 1 lower part. 4-Methylpyrazole (0.084 mmol/kg) was given 15 min. before ethanol (32.6 mmol/kg), n = 24 The rate of elimination decreased from 6.6 to 1.9 µg ethanol per ml per min. The decline showed a curvilinear course. With higher doses the degree of inhibition was larger The degree of inhibition was calculated from values for min_{-0} (time in min. until blood ethanol concentration equalled

Table 1

Effect of 4-methylpyrazole on *blood ethanol parameters* in the rat. 1.5 g/kg ethanol intraperitoneally with or without 10 mg/kg 4-methylpyrazole, t-test, df 23

Blood ethanol parameters	Ethanol	Ethanol + 4-methyl-pyrazole	t	P
Time until blood ethanol zero (min.)	229 ± 6.0	749 ± 14.0	33.2	< 0 001
C_0 (mg ethanol per ml blood)	1.81 ± 0.03	1 72 ± 0.03	1.7	> 0.05
$\beta \times 10^3$ (µg ethanol per ml blood per min.)	8.0 ± 0.26	2.3 ± 0.06	20.0	< 0.001
$\beta r \times 10^3$ (µg ethanol per kg per min.)	6.6 ± 0.79	1.9 ± 0.2	26 8	< 0.001
(dose/C_0)	0.83 ± 0.01	0.88 ± 0.02	2.1	> 0.05
maximal ethanol conc. (mg/ml blood)	1.63 ± 0 03	1.69 ± 0.03	1.2	> 0.05

zero, extra-polated *from the declining part of the blood ethanol curve)* according to the formula

$$\text{Degree of inhibition} = 100 \; \frac{^{min}(4\text{-MP} + \text{EtOH}) \;\; ^{min}\text{EtOH}}{^{min}(4\text{-MP} + \text{EtOH})} \quad (1),$$

where ^{min}(4-MP + EtOH) is the time until the blood ethanol concentration equalled zero when 4-methylpyrazole was given together with ethanol, and ^{min}EtOH the time until the blood ethanol concentration equalled zero when ethanol alone was given. In fig. 2 the results are shown when doses of 0 0053 − 3.0 mmol 4-methylpyrazole per kg body weight were administered. A 50 / inhibition was brought about by a dose of 0 017 mmol/kg. With 0 084 mmol/kg the degree of inhibition was 69 4 / The maximal degree of in hibition was 80 / and was achieved with a dose of 0.34 mmol/kg.

Role of length of interval between 4-methylpyrazole and ethanol.

In rats 32.6 mmol ethanol per kg body weight was administered at certain intervals (15 min. − 48 hrs) after 4-methylpyrazole, 0 084 mmol/kg, and the elimination of ethanol from the blood was studied. The degree of inhibition was calculated according to formula (1). The results are given in table 2. The inhibitory effect of 4-methylpyrazole on ethanol metabolism persisted for a few hours only and then declined.



Fig. 2. Degree of inhibition of the elimination of ethanol (32.6 mmol/kg) after various doses of 4-methylpyrazole.

Influence of 4-methylpyrazole on ethanol-induced incoordination.

1 Long-term control group (saline + saline n = 8) The mean tilting angles in the initial measurements were on an average 53 6 ± 0 68 degrees. In the various trials performed one week apart the initial values were 53.6, 54 0 51.5 51.3 and 48.8 degrees, thus indicating a slight decrease with time. The values showed a variation during the day in "week 1 of maximally 4 9 / (fig. 3) and in "week 5" of 2.0 / the differences not being significant ($P > 0.5$)

2 Groups with ethanol and/or 4-methylpyrazole The pre-drug values in "week 1" (n = 24), given in fig. 3 did not differ from those obtained in

Table 2

Duration of the inhibitory effect of 4-methylpyrazole on ethanol elimination.

Interval between the administration f 4-methylpyrazole and of ethanol (hrs)	Degree of inhibition of ethanol elimination (per cent)
0.25	67
1	61
	46
4	14
12	−10
24	8
48	−3

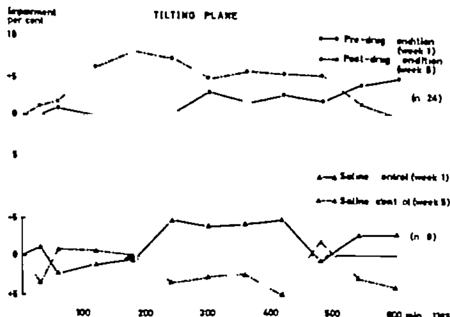


Fig. 3. *Upper part:* Changes in degree of impairment with time in the drug series, in the pre-drug condition ("week 1") and in the post-drug condition ("week 5"). *Lower part.* Changes in degree of impairment with time in the control series (saline + saline for five weeks), "week 1 and "week 5"

the long term saline controls (n = 8). *Ethanol* induced a maximal impairment of 11.9 / which occurred simultaneously with the maximal blood ethanol concentration. The impairment then decreased in parallel with the decline in blood ethanol concentration (fig. 1), and was detectable for 300 min. *4-Methylpyrazole* caused a delayed maximal impairment of 7.2 / at 180 min. which was normalized after about 9 hrs. *Ethanol + 4-methyl pyrazole* brought about a maximal impairment of 14.9 / exceeding that of ethanol or 4-methylpyrazole alone. The impairment then decreased mainly in parallel to the decrease in blood ethanol concentration. After 540 min. no impairment could be detected.

The addition of 4-methylpyrazole thus both prolonged and increased the ethanol-induced incoordination.

3 Statistical comparison between conditions Intra-individual differences of performance recorded between the control and the drug conditions were submitted to two-tailed t-tests, and to analysis of variance. Significant differences were obtained (a) between ethanol and control during the five first determinations, the significance of the difference ranging from $P < 0.05$ to $P < 0.001$ df = 23 (b) between 4-methylpyrazole and control in all ..

between 120 and 360 min. ($P < 0.05$ to $P < 0.001$) and (c) between ethanol + 4-methylpyrazole and control in all trials up to 420 min., the significances of the difference being $P < 0.001$

Within the various drug conditions, no consistent differences could be found between the 3 different sub-groups, when tested by analysis of variance.

The mean performance scores, i. e. the degree of impairment corrected for the values found in the control condition, were plotted against the corresponding mean values of blood ethanol concentration, for the ethanol condition as well as the ethanol + 4-methylpyrazole condition, starting with the peak values. Rectilinear regression lines were found. The equation for the ethanol condition was $y = 14.83 + 5.05\ x$, and for the ethanol + 4 methylpyrazole condition $y = 34.80 + 6.70\ x$. The slopes did not differ significantly ($P > 0.05$) either when tested for all three subgroups ($n = 24$) or for only the group tested on the first week ($n = 8$). However the degree of impairment was significantly higher in the ethanol + 4-methylpyrazole condition than in the ethanol condition for a certain ethanol level. The regression lines were significantly different, either when tested between the values of the first week and control ($df = 1/3$ $F = 62.35$ $P < 0.001$), or between the scores in all three weeks ($df = 1/3$ $F = 6.98, P < 0.05$), and when tested by analysis of covariance. Thus, in the ethanol + 4-methylpyrazole condition, the degree of impairment was greater than that found with the prolonged high ethanol concentration.

4 Area of Impairment As an expression of the over-all effect, the area of impairment (per cent impairment x minutes) was calculated plani-metrically *The pre-drug control condition corresponded to an area of* 1050 units. When corrected for control values, the areas were 665 units in the ethanol condition, 840 units in the 4-methylpyrazole condition and 3640 units after ethanol + 4-methylpyrazole.

5 Post-drug condition. In the post-drug animals, initial tilting values were lower than in earlier conditions, 47.9 degrees against 56.2 degrees in "week 1" (fig. 3). In addition, a more severe impairment after 8.4 ‰ of saline was seen in the post-drug condition than in the pre-drug condition. Intra-individual differences were submitted to two-tailed t-tests. *Significant* differences were obtained at 120–420 min. between "week 5" and "week 1 ranging from $P < 0.05$ to $P < 0.001$

The performance curves in the "post-drug" condition were also compared with those from the long-term control series, when only saline + saline was administered five times. The mean values of the consecutive measurements in the "post-drug" condition were compared by t-tests with the corresponding values *from* "week 5 *obtained* in the control series. Two of the differences obtained were significant ($P < 0.05$)

Discussion

A survey of the pharmacology of pyrazole compounds was published by ORTH (1968). Since pyrazole has considerable adverse effects on the experimental animals tested, there has been a search for analogues giving a similar competitive inhibition of LADH (EC 1 1 1 1), but with a minimum of adverse or toxic effects. A large number of pyrazole derivatives have been tested for their LADH-inhibiting potency (LESTER et al. 1968 LI & THEORELL 1969 THEORELL et al 1969 REYNIER 1969) Substitution with iodine bromine or methyl in the 4-position of pyrazole enhanced the inhibitory effect, while substitution in positions 1 or 3 removed the inhibitory activity Subacute toxicity studies of 4-methylpyrazole in rats and in dogs (E. HANSSON, G MAGNUSSON & N -O BODIN, personal communication 1969) comprising blood chemistry haematology urine analysis and histopathology did not reveal any toxic effects in the doses used (0 084 – 1.7 mmol/kg) for up to 4 weeks.

The present investigation confirms the results obtained by BLOMSTRAND & THEORELL (1970) and BLOMSTRAND (1971) indicating that 4-methylpyrazole is an active inhibitor of ethanol elimination *in vivo* When calculated from data of times until the blood ethanol concentration equalled zero a 50 / inhibition of ethanol elimination (32.6 mmol/kg) was elicited by a dose of 4-methyl-pyrazole of 0 024 mmol/kg. However since the inhibition of ethanol elimination by 4-methylpyrazole is competitive (REYNIER 1969) and 4-methylpyrazole is eliminated more slowly than ethanol (RYDBERG et al. 1972), the degree of inhibition varies with time.

The duration of the inhibitory action of 4-methylpyrazole on ethanol elimination is limited to a few hours (table 2) This is in accordance with studies on the elimination of 4-methylpyrazole from the blood in the rat (RYDBERG et al. 1972) A half-life time of 9 hrs was found. When ethanol was also given, the half-life time significantly increased to 13 hrs. GOLDSTEIN & PAL (1971) have determined a half life time of 10 hrs for pyrazole and of 3 hrs for 4-bromopyrazole in the mouse.

4-Methylpyrazole reduces a number of ethanol-induced metabolic disturbances. The ethanol-induced fatty infiltration of the liver in rats was partially inhibited by 4-methylpyrazole, when ethanol (98–174 mmol/kg) and 4-methylpyrazole (1.7 mmol/kg) were administered (BLOMSTRAND & FORSELL 1971). From their graph it could be deduced that the liver triglycerides decreased from 16.6 mg/g liver in the ethanol-treated animals to 8.9 mg/g when 4-methylpyrazole was given. The control values were 5.2 mg triglycerides per g of liver The ethanol-induced increase in the lactate/pyruvate ratio was almost normalized in human subjects when 4-methylpyrazole w given (BLOMSTRAND & THEORELL 1970)

A number of ethanol-induced metabolic disturbances are reduced by pyrazole (LESTER *et al.* 1968 BUSTOS *et al.* 1970 JOHNSON *et al.* 1971) as well as by 4-methylpyrazole (BLOMSTRAND & THEORELL 1970 BLOMSTRAND & FORSSELL 1971) Certain differences are obvious, however when the effects of pyrazole and of 4-methylpyrazole are compared. The K_i values for 4-methylpyrazole is 1.2 μM as against 4.2 for pyrazole (REYNIER 1969). A 50 / inhibition of ethanol elimination *in vivo* was elicited by pyrazole (0.30 mmol/kg) according to GOLDBERG & RYDBERG (1969) as against 0.024 mmol/kg for 4-methylpyrazole (BLOMSTRAND & THEORELL 1970 RYDBERG & NERI this paper) Toxic effects on bone marrow hepatic and renal function have been reported for pyrazole (WILSON & BOTTIGLIERI 1962), whereas 4-methylpyrazole did not give any toxic effects in doses of 0.084 – 1 7 mmol/kg (loc. cit.). The presence of pyrazole in blood samples may disturb enzymatic determinations of ethanol, due to an inhibition of the yeast alcohol dehydrogenase used in the assay (GOLDBERG & RYDBERG 1969 RYDBERG 1971), while 4-methylpyrazole lacks any such effects (this paper).

The effects of enzyme inhibitors *in vivo* may be considerably different from those obtained under *in vitro* conditions. 4-Iodopyrazole was reported to have a K_i value one tenth that of pyrazole for an inhibition of rat LADH *in vitro* (REYNIER 1969) while no significant difference in the inhibition of ethanol elimination *in vivo* could be found between pyrazole and 4-iodopyrazole (RYDBERG 1969) 4-Methylpyrazole, on the other hand, is relatively more active *in vivo* than *in vitro* when compared to pyrazole.

Whereas certain ethanol-induced metabolic disturbances are reduced by pyrazole, pyrazole acts synergistically with ethanol on the central nervous system (BLUM *et al.* 1971 GOLDBERG & RYDBERG 1969 GOLDBERG *et al.* 1972). This is partly explained by the prolonged presence of ethanol in the blood and brain after the administration of pyrazole. However pyrazole alone gives a behavioural depression (GOLDBERG *et al.* 1972).

The present investigation has shown that 4-methylpyrazole also induces a behavioural depression *per se* and, moreover acts synergetically with ethanol producing both an enhancement and a prolongation of the disturbances of coordination induced by ethanol, exceeding that corresponding to the prolonged high concentration of ethanol.

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From the Research Laboratories of H. Lundbeck & Co. A/S, Ottiliavej 7-9
DK 2500 Valby Copenhagen, Denmark

Increased Specificity of the ^{3}H Acetic Anhydride Coupling Method for Plasma Analysis of Drugs Containing Secondary Amino Groups

By

K. Fredricson Overø

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Abstract For the plasma analysis of drugs containing primary or secondary amino groups the method of HAMMER & BRODIE (1967) has been widely used. By this technique amines are isolated by extraction and coupled with ^{3}H-acetic anhydride. After removal of excess labelling agent, the amount of labelled coupling product is assayed by liquid scintillation counting. The possibility of masking primary amines by adding salicylic aldehyde to the hexane phase in the first extraction step of this method has been investigated. The presence of 0.1 M salicylic aldehyde was found t reduce considerably the amount of primary amine measured as ^{3}H-amide, while secondary amines were only slightly influenced. Thus increased specifity for secondary amines was achieved. The practical importance of the modified method was illustrated by experiments on plasma from rats given nortriptyline and human subjects given Lu 5-003, bicyclic thymoleptic. In the former case the modified method is of less importance, since no primary amine metabolite is present – in the latter case, however it would be advantageous, because the corresponding primary amine is present as metabolite in plasma.

Key-words: Thymoleptics – kinetics – radioisotopes – nortriptyline.

The ^{3}H-acetic anhydride coupling method of HAMMER & BRODIE (1967) has been widely used for the assay of various drugs in plasma. The principles of the method is outlined in fig. 1. Since this technique may be used for the assay of secondary as well as primary amines, the specificity of the method may be questioned especially in cases where a secondary amine is assayed and where the corresponding primary amine might be present as a metabolite. This problem was discussed by HAMMER & BRODIE (1967) in their work on desmethylimipramine and has also been commented upon in other papers on desmethylimipramine and nortriptyline (HAMMER *et al* 1966 SJÖQVIST *et al.* 1969 BORGÅ & SJÖQVIST 1969). It is concluded, however that with these drugs the amounts of primary amine are presumedly too small to interfere to any notable extent.

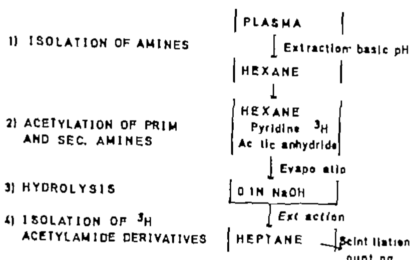


Fig. 1. The principles of the HAMMER & BRODIE (1967) coupling technique.

When performing pharmacokinetic studies with the thymoleptic substance Lu 5-003 however it was discovered that human plasma samples contained not only Lu 5-003 which is a secondary amine but also small amounts of the corresponding primary amine as a metabolite. For this reason the possibility of modifying the method in order to obtain an increased specificity for secondary amines was investigated. These investigations are described in the present paper

Materials and Methods

The assay of secondary and primary amines was performed according to the technique described by HAMMER & BRODIE (1967).

In order to investigate whether it would be possible to mask primary amines by coupling them with salicylic aldehyde as proposed by NYBERG (1970), a series of experiments was performed with salicylic aldehyde added to the hexane phase in the first extraction step Apart from this modification and the fact that the samples were ex tracted for 20 instead of 10 minutes, the procedure was exactly as described by HAMMER & BRODIE (1967)

In order to investigate what coupling products were obtained, thin-layer chromatography (TLC) was performed on the remainder of the heptane phases after removal of aliquots for direct liquid scintillation counting. The remainder of the heptane phase was evaporated to dryness, redissolved in a small volume of chloroform and quantitatively applied on silica gel plate. A benzene:acetone:diethylamine mixture (80:20:1) was used as solvent. After development the silica gel of the plate was scraped off for each half centimeter by means of an automatic device. The radioactivity of these frac tions was measured directly by liquid scintillation counting in the dioxane-methanol-toluene-naphthalene mixture (diotol) described by HERBERG (1960) and modified by replacing POPOP with dimethyl POPOP Graphs for radioactivity per fraction versus

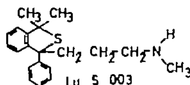


Fig. 2. Formula of Lu 5-003.

Rf-values were constructed to illustrate the contents of coupling products.

The following compounds were assayed: nortriptyline (NT), N-desmethylnortriptyline (Lu 5-079), Lu 5-003 (a secondary amine; for formula see fig. 2) and Lu 5-060 (the primary amine corresponding to Lu 5-003).

Plasma samples were obtained from rats given three oral doses of 100 mg/kg nortriptyline (at –24, –16 and 0 hours. Samples were taken 1 and 2 hours after the last dose) and from patients after infusion of 60 mg Lu 5-003, a bicyclic thymoleptic.

Results

The amount of primary amine reacting with labelled acetic anhydride is considerably reduced by the addition of 0.05 M and even more by 0.1 M salicylic aldehyde (fig. 3). The counting efficiency was not influenced by the

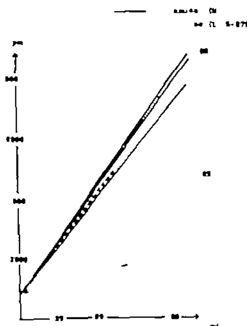


Fig. 3. Standard curves without (O) and with salicylic aldehyde (0.05 M and 0.1 M).

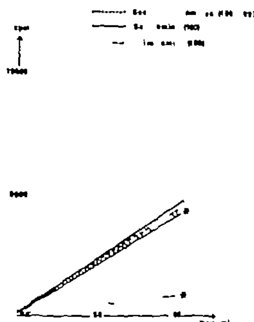


Fig. 4. Standard curves with (*) and without salicylic aldehyde. Amines. Lu 5-003 and Lu 5-060.

addition of salicylic aldehyde – hence cpm values are depicted. The assay of secondary amine is influenced only to a minor extent. Even with 0.1 M salicylic aldehyde small amounts of primary amine remain free. However since a higher concentration of salicylic aldehyde was found to cause disturbances in the procedure by not being fully evaporable and giving high blind values, 0.1 M salicylic aldehyde was chosen for further experiments. One of these (with Lu 5-003 and Lu 5-060) is shown in fig. 4. A mixture of equal amounts of secondary and primary amine is seen to give approximately the same standard curve as secondary amine alone, when the modified method is used. In a series of nine experiments with the two amine pairs the amount of primary amine was reduced by 84 ± 5 / (mean ± S.D.) when salicylic aldehyde was added, i.e. 100 ng/ml of primary amine resulted in a cpm value that was approximately twice that of the blind value. In this respect no difference between the two primary amines was seen.

These observations were further confirmed by TLC on the heptane extracts after assay of secondary amine, primary amine and a mixture of equal amounts of secondary and primary amine. Fig. 5 shows the picture for NT/Lu 5-079. The picture for Lu 5-003/Lu 5-060 was very similar. The peak corresponding to the labelled coupling product of primary amine is seen to be almost eliminated when the modified method is used. The peak corresponding to the secondary amine, however, is only slightly influenced. Thus

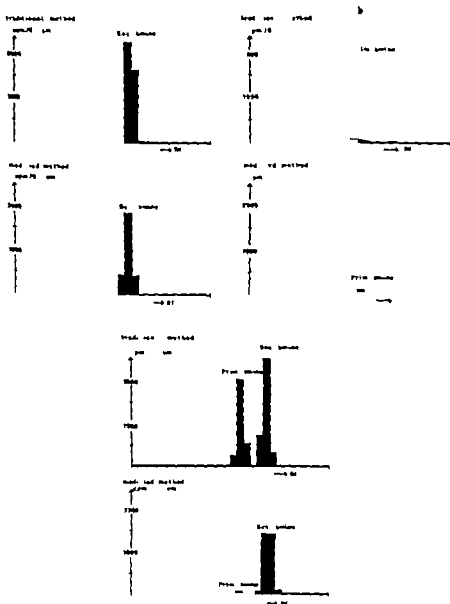


Fig. 5. TLC of heptane extracts after assay of a secondary amine (a), a primary amine (b) and a mixture of the two (c). Amines: NT and Ln 5-079

the TLC picture corresponds very well with the standard curve pattern. The results do not indicate any differences between the coupling products of primary and secondary amines as regards recovery from the silica gel. Such differences would not be expected since the chemical properties of the amines (especially in coupled form) are very similar and since they are readily soluble in diotol. Therefore the size of the peaks may be concidered to be an estimate of the amounts of amine present.

Thus a more specific assay of secondary amine seems to be achieved by this very simple modification of the method. To illustrate the practical applications of the modified method, the results of some experiments with plasma from dosed animals will be described.

In the first of these, assays of nortriptyline in rat plasma performed both with the traditional and the modified technique were compared. The samples proved to contain only minute amounts of the primary amine metabolite (fig. 6) and no actual difference between the methods is seen. However some unknown products (which have also been observed in some samples of control

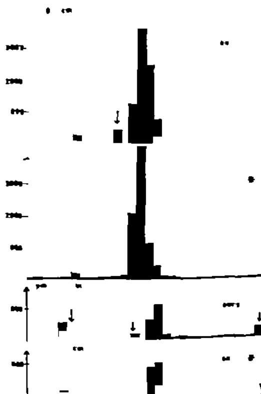


Fig. 6. TLC of extracts after assay of plasma from rats given nortriptyline (1 and 2 hour samples): traditional and modified (*) method.

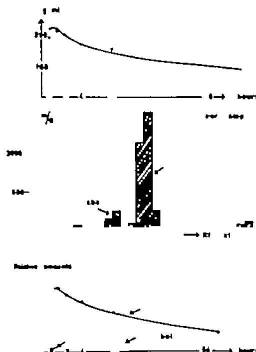


Fig. 7 Plasma concentration after infusion of Lu 5-003 into man, traditional method only

plasma) are removed by the modified technique, which must be considered an additional advantage.

Finally the experiments with plasma samples from patients given Lu 5-003 which initiated the search for a more specific method, are illustrated in fig. 7 These samples were assayed by the traditional technique, and TLC of the coupled sample extracts revealed the presence of two peaks, the Rf values of which corresponded to the labelled coupling products of Lu 5-003 and the primary amino metabolite respectively Separate plasma curves for drug and metabolite could be constructed based on the amounts of radioactivity in the two peaks.

Discussion

No practical difficulties are encountered in the method with the suggested modification. Plasma control values were often found to be somewhat lower than with the traditional method. For these reasons salicylic aldehyde might well be added routinely in all experiments, when a specific assay is wanted and when primary amines – as metabolites or from other sources – might be present. However since standard curves obtained by the modified

are generally somewhat lower than those obtained by the traditional method, the sensitivity of the method might be somewhat lowered by the addition of salicylic aldehyde.

As for the practical importance of the modification, preliminary experiments indicate that for the assay of Lu 5-003 in human plasma after infusion of the drug, the modified method would have been very useful for masking the metabolite. For the determination of nortriptyline in rat plasma, the modification is of no advantage with regard to the removal of the metabolite. However small amounts of unknown coupling products, which would otherwise have been estimated as drug are removed.

It may be concluded that the practical importance of a more specific method may vary with several factors such as the drug, the mode of administration and the species. Moreover the requirements for specificity are dependent on what kind of study the assay is needed for. For pharmacokinetic studies with secondary amines which metabolize to primary amines, and in cases where primary amines from other sources are present, the modified method would be advantageous.

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From the Department of Pharmacology University of Uppsala,
Uppsala, Sweden

Hexoestrol Analogues as Probes of Oestrogen Receptors
I. Importance of Hydrogen-Bonding Groups for Receptor Binding

By

Lars Terenius

(Received February 10, 1972, Accepted March 17 1972)

Abstract Analogues of the synthetic oestrogen *meso*-hexoestrol with systematically varied *p,p'*-substituents were prepared. The competitive affinity of these analogues for the soluble oestrogen "receptor" molecules from mouse uterus were then measured. The results showed that the *p,p'*-hydroxyls of *meso*-hexoestrol are very important for affinity. Substitution of one of the hydroxyls as in the OH,NH_2, OH,CH_2OH, OH,OCH_3, OH,F or OH,H analogues lessened the binding affinities 15 to 90% while the OH,COOH analogue had < 99 % the binding affinity of *meso*-hexoestrol. Replacement of both hydroxyls of hexoestrol as in the H,H, OCH_3,OCH_3, CH_2OH,CH_2OH, NH_2,NH_2, SH,SH or $NHSO_2CH_3$, $NHSO_2CH_3$ analogues led to very marked reduction in affinity (> 99.9 %). Hydrogen-bonding interchange at two spatially well-defined sites seems to attach the oestrogen to the receptor. One of these bonds evidently has a higher energy of formation than the other.

Key-words: Hexoestrol – oestrogens – uterus – receptors – hydrogen-bonding.

Selective oestrogen receptor molecules capable of binding oestrogens have been isolated from target tissues (DeSombre *et al.* 1969, Gorski *et al.* 1970). (The oestrogen-binding proteins are here called receptors for convenience. Their functional significance remains to be proved.) Although not conclusively established, much evidence supports the assumption that these receptive molecules can either be considered as the receptors themselves or else form the binding components of these receptors. For instance, the present author has shown a structural and optical specificity of the binding sites in the mouse uterus which is in accordance with that expected from the bioassay data (Terenius 1966, 1967 & 1968). Work by others (Brecher & Wotiz 1968, Korenman 1969) on the binding characteristics of the cytoplasmic receptor molecule from the rat uterus supports this assumption. However these published studies do not give much information about the chemical

anatomy of the binding sites because of the fairly unsystematic choice of substances.

This study present receptor affinities of compounds related to the synthetic oestrogen *meso*-hexoestrol in which the p p'-substituents were systematically varied. Hexoestrol is an ideal oestrogen for this type of study since it has a symmetrical and comparatively simple structure.

Materials and methods

A. Chemicals.

Syntheses.

All the tested derivatives of *meso*-3,4-diphenylhexane (table 1) were prepared by the author. Compounds previously described in the literature had melting points close to those previously reported. Ir spectra of most compounds were obtained and bonds (or peaks) were as expected. The nmr spectra of several of the compounds were recorded, including all the new ones, these were also as expected.

erythro-3-(p-methoxyphenyl)-4-(p-hydroxyphenyl)hexan (R=OH, R'=OCH_3).

meso-Hexoestrol was methylated with an equimolar amount of dimethyl-sulphate according to routine procedures. The mono-methylether was precipitated from an alkaline salt solution. The precipitate was repeatedly crystallized from ethanol/water ethylene chloride, and benzene. The product was free from starting material and dimethylether as shown by TLC.

erythro-3-(p-hydroxymethylphenyl)-4-(p-hydroxyphenyl)hexane (R=OH, R'=CH_2OH).

This compound was obtained by reduction of the corresponding acid (Wilson *et al.* 1953) (0.004 mol) with $LiAlH_4$ (0.025 mol) in 15 ml of anhydrous ether at reflux temperature for 3 hours. Isolation in the usual way gave the desired product in a 60 % yield. It was recrystallized from benzene. Infrared spectrum and TLC showed absence of carboxyl group.

meso-3,4-bis-(p-hydroxymethylphenyl)hexane (R=R' = CH_2OH).

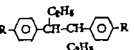
Chloroacetyl chloride (0.15 mol) was allowed to react with *meso*-3,4-diphenylhexane (0.05 mol) in the presence of anhydrous $AlCl_3$ (0.24 mol) in carbon disulphide (160 ml) according to the usual Friedel-Crafts procedure. A 90 % yield was obtained of *meso*-3,4-bis(p-chloroacetylphenyl)hexane, m.p. 193–195 (from benzene/cyclohexane). This compound was oxidized in dilute sodium hypochlorite solution according to Wilson *et al.* (1953). The product, *meso*-3,4-bis-(p-carboxyphenyl)-hexane, crystallized from 70 % acetic acid, m.p. > 300. Yields in this step were about 70 %. The above product (0.005 mol) was reduced with $LiAlH_4$ (0.1 mol) in anhydrous ether. The desired product crystallized from dioxan/cyclohexane in 55 % yield.

meso-3,4-bis-(p-methanesulphonamidophenyl)hexane (R=R'=$NHSO_2CH_3$).

0.004 mol of diamino-compound (R=R'=NH_2) was mixed with methane-sulphonyl-chloride (0.009 mol) in ether (20 ml). Five ml of 2 N-NaOH was added drop-wise under vigorous stirring. After standing for 1 hour the solution was poured into water and extracted with butanol. The product was crystallized from butanol and benzene/cyclohexane in 40 % yield. The synthesis of this compound by a similar procedure was recently reported (Mikolasek *et al.* 1969).

Table 1

Derivatives of *meso*-3,4-diphenylhexane.



Substituents R	R	Mol.wt.	m.p. C	Prepn. method (ref.)	Calcd., % C	H	N	S	Found, % C	H	N	S
H	H	238.4	92.5–94	Wawzonek (1946)	78.5	6.6			78.4	6.6		
H	OH	254.4	142.5–144	Wawzonek (1946)	85.0	8.7			85.0	8.3		
OH	OH	270.4	186–187	Wilds & McCormack (1949)	80.0	8.2			80.0	8.2		
OH	OCH_3	284.4	118.5–120	*b*	80.2	8.5			80.1	8.3		
OCH_3	OCH_3	298.4	144.5–146	Wilds & McCormack (1949)	80.4	8.8			80.3	8.7		
OH	COOH	293.4	169–171	Wilson *et al.* (1953)	76.5	7.4			76.3	7.3		
OH	CH_2OH	284.4	164–166	*b*	80.2	8.5			79.6	8.5		
CH_2OH	CH_2OH	298.4	183–184.5	*b*	80.5	8.8			80.1	8.7		
OH	F	272.4	129–130.5	Wilds & McCormack (1949)	79.4	7.8			79.9	7.8		
OH	NH_2	269.4	185–187	Buckerstaff & Wilds (1949)	80.3	8.6	5.2		80.6	8.6	5.2	
NH_2	NH_2	268.4	141–142	Huang-Minlon (1948)	80.6	9.0	10.4		80.5	8.8	10.3	
SH	SH	302.4	147–150	Terenius (1970)	71.5	7.3		21.2	71.6	7.4		21.1
$HNSO_2CH_3$	$HNSO_2CH_3$	458.4	246–249	*b*	56.6	6.7	6.6		56.6	6.7	6.6	

Melting points are uncorrected.
b See Methods.

Labeled oestradiol-17β Tritium-labeled oestradiol 17β (6,7-³H) was purchased from NEN Chemicals GmbH, Dreieichenhain, Germany The specific activity was 40 ci/mmol. On thin-layer chromatograms 95 to 98 % of the radioactivity moved as single entity with authentic oestradiol-17β.

B Receptor binding experiments

Uteri excised from 20 to 25 g N.M.R.I. mice were cleaned of adherent fat, finely minced and homogenized in 3 volumes of a 0.01 M Tris buffer pH 7.4, with 0.0015 M EDTA and 0.25 M sucrose. All manipulations of the tissues were performed at cold room temperature. Homogenization was done with a Polytron PT 10 (Kinematica, Luzern, Switzerland) giving 3 times 5-second pulses at setting 5 with 2 minutes cooling in between in an ice bath. The homogenate was centrifuged for 1 hour at 129,000 × g_{av}. To avoid the fat floating to the top the centrifuge tubes were punctured just above the pellet and the supernatant obtained by gravity flow The supernatants contained about 10 mg protein/ml (according to the Lowry method with bovine albumin as standards) and about 5 pmol receptor sites/ml.

Tests for competitive binding affinities were done in 3 ml centrifuge tubes containing 0.5 ml of the buffer cited above and hormones. In a typical experiment the competitors were tested at fixed concentrations and were allowed to compete with 5 different concentrations (3×10^{-11}M, 9×10^{-11}M, 2×10^{-10}M, 5×10^{-10}M, 1.4×10^{-9}M) of labeled oestradiol-17β. Control tubes with oestradiol-17β only were run simultaneously For each competitor or control group 4 tubes per concentration were run, i.e., each group consisted of 20 tubes. To the tubes so prepared 25µl of the high speed uterus supernatant was added. The tubes were then vortexed and allowed to stand at +4 for 16 hours. 0.5 ml of the charcoal suspension (0.05 % Dextrane 10 from Pharmacia, Uppsala, Sweden and 0.5 % Norite A from Sigma Co. St. Louis, Mo.) was added to the tubes which were vortexed, allowed to stand for 10 minutes and centrifuged for 10 minutes at 3000 × g_{av}. Of the supernatant, 0.5 ml was withdrawn, mixed with 5 ml of scintillation solution (3 l dioxan, 300 ml methanol, 375 g naphthalene, 12 g PPO, 0.15 g dimethyl POPOP) and the radioactivity was measured in a liquid scintillation counter

The reciprocal of bound oestradiol-17β was plotted against the reciprocal of total oestradiol 17β in the test tube. The regression lines of these plots for the different compounds were obtained by a computer programme using least squares.

Results

The *meso*-hexoestrol analogues were tested for their ability to inhibit the binding of oestradiol 17β to the binding molecules in mouse uterus cytosol. The relative affinities of the analogues were calculated in reciprocal plots (EDSALL & WYMAN 1958) analogous to the Lineweaver – Burk plots. These plots should give straight lines if the underlying assumptions are valid. Furthermore, if the inhibition is competitive in nature the slope of the line is increased by the presence of the inhibitor but the intersection with the ordinate axis remains constant. However the situation is complicated by the fact that allosteric or other conformational interference gives competitive" plots. Linearity prevailed in all plots. The slopes of the regression lines had stan-

dard errors of less than 10 / in almost every case the few exceptional cases were not used for calculations. The results of all plots were also in favour of a competitive interaction. The results of the plots were used to calculate inhibition constants (table 2)

It was found that *meso*-hexoestrol itself had the highest affinity for the receptor Substitution of one hydroxyl by NH_2, CH_2OH OCH_3 or F caused

Table 2

Inhibition constants of *meso*-hexoestrol analogues. The means and the individual values of 2 or 3 experiments are given.

R–⟨O⟩–CH(C_2H_5)–CH(C_2H_5)–⟨O⟩–R R	 R	K_i (M)	Relative molar affinities (K_i (R=OH R=OH)/K_i)
OH	OH	9.4×10^{-11} (1.5×10^{-10} 6.3×10^{-11} 7.0×10^{-11})	1
OH	NH_2	1.1×10^{-10} (8.1×10^{-11} 1.4×10^{-10} 1.1×10^{-10})	0.85
OH	CH_2OH	1.5×10^{-10} (1.5×10^{-10} 1.6×10^{-10})	0.63
OH	OCH_3	1.4×10^{-10} (1.5×10^{-10} 1.4×10^{-10})	0.67
OH	F	3.1×10^{-10} (6.1×10^{-10} 1.7×10^{-10} 1.6×10^{-10})	0.30
OH	H	7.8×10^{-10} (5.8×10^{-10} 1.3×10^{-9} 4.5×10^{-10})	0.12
OH	COOH	1.1×10^{-8} (8.6×10^{-9} 1.3×10^{-8})	0.0085
NH_2	NH_2	1.1×10^{-7} (1.3×10^{-7} 8.8×10^{-8})	0.00085
CH_2OH	CH_2OH	5.2×10^{-7} (6.2×10^{-7} 4.1×10^{-7})	0.00018
H	H		–
OCH_3	OCH_3		–
SH	SH		–
$NHSO_2CH_3$	$NHSO_2CH_3$		

– No inhibition at 10^{-4} M

a moderate decrease in affinity while mono-substitution with COOH reduced the affinity to less than 1 / of that in the parent compound. Replacement of both phenolic hydroxyls led to very low affinity or to inactivity under the test conditions. It should be noted that particularly the H,H and the SH,SH analogues are less hydrophilic than the other derivatives tested. It is therefore possible that they were not completely in solution at the indicated concentration.

Discussion

meso-Hexoestrol has two hydroxyls, which like those in oestradiol-17β are separated by about 15 A. These hydroxyls, which are hydrogen bonding groups are considered to be crucial for the interaction between oestrogens and a receptor (GRUNDY 1957) In the present communication the importance of these groups in the oestrogen-receptor interactions has been studied. Therefore "minimum-deviation" analogues were prepared, i.e., substances with isosteric, isoelectronic or bioisosteric replacement of the hydroxyls.

Replacement of *one* hydroxyl in hexoestrol by other groups did not markedly decrease the binding affinity When R was NH_2, CH_2OH, OCH_3 or F the affinities were more than 30 / of that in the original compound but 2–9 times higher than with H, indicating that the former groups contributed to binding, probably as hydrogen *acceptors* (since F and OCH_3 contribute). It should be noted that although F and OH are isosteric they act differently on electrons in the benzene ring. Such factors may also be of some importance. It is also clear that the receptor sites can accomodate larger *p*-substituents (CH_2OH) without much change in affinity Substitution with carboxyl (COOH) caused a drastic reduction in affinity (100 times). This was unexpected since it has been found earlier that oestrogens with one carboxyl can be firmly bound to the receptors. For instance, demethylated fenocyclin has about the same competetive binding affinity as *meso*-hexoestrol (TERENIUS 1967).

The essential role of *one* phenolic hydroxyl for receptor affinity is illustrated by the fact that the diamino and dimethylenehydroxy analogues had very low affinities (1/1000 and 1/5000 of hexoestrol respectively). In spite of hydrogen bonds with amino or hydroxyl groups having the same energy of formation (PIMENTAL & MCCLELLAN 1960) they are not equivalent in this case. The basic character of the nitrogen may be one reason, although at the pH of the buffer 7 4 ionization is suppressed ($pK_a \sim 4.6$). The CH_2OH group provides the hydrogen-donating hydroxyl on the smallest possible carbon side-chain. This group has small effects on electrons in the benzene ring but the aliphatic hydroxyl is as strong a hydrogen-donor as the phenolic hydroxyl (PIMENTAL & MCCLELLAN 1960). It is possible that the very low

affinity of the disubstituted compound (R=R =CH_2OH) could be due to an unfavorable steric orientation of the hydroxyl(s)

The total absence of hydrogen donating groups as in the hydrocarbon, in the dimethylether and in the methanesulphonamido analogue led to the loss of measureable affinities. The methanesulphonamido analogue (R=R = $HNSO_2CH_3$) was prepared since, in a number of phenethanolamines, this group has been found to be bioisosteric with the phenolic hydroxyl with re tention of biological activity (LARSEN & LISH 1964) Such replacement in hexoestrol led to loss of oestrogenicity thus confirming MIKOLASEK *et al.* (1969), and to inactivity *in vitro* The dithiol analogue was also devoid of affinity Since sulphur forms very much weaker hydrogen bonds than oxygen (PIMENTAL & MCCLELLAN 1960) this also indicates the importance of hydrogen bonding. The somewhat greater atomic radius of sulphur is probably not responsible for the inactivity since the binding sites can evidently accomodate one methoxyl group or two methylenehydroxy groups which are larger

The present data give some indications as to the factors which are responsible for attachment to the receptor *One* hydroxyl in the hexoestrol molecule is extremely important while the other can be replaced by amino methylenehydroxy or methoxy without much loss in affinity Absence of a second hydroxyl or its replacement by fluorine gives lower affinities but they are still over 10 / of that of the parent molecule. Evidently two hydrogen-bonds anchor the hexoestrol molecule to the receptor surface, one of these having a much higher energy of formation. The hydrogen bond forming groups of the receptor could, with regard to the proteinaceous nature of the binding molecules (DESOMBRE *et al* 1969 GORSKI *et al.* 1970), either be the hydroxyls in serine or threonine or the imidazole moiety of histidine. It is interesting to note that PUCA & BRESCIANI (1970) who observed that iodine treatment inactivates the receptor consider histidine or tyrosine to be at or near the receptor site.

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From the Department of Pharmacology University of Uppsala,
Uppsala, Sweden

Hexoestrol Analogues as Probes of Oestrogen Receptors II. Importance of Hydrogen-Bonding Groups for Binding to Uterine Tissue and for Uterotrophic Activity

By

Lars Terenius

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Abstract. Analogues of *meso*-hexoestrol with variation in *p*,p'-substituents (TERENIUS 1972) were tested with regard to 1) competitive affinity for oestrogen receptors in intact uterine tissue *in vitro* 2) uterotrophic and 3) anti-uterotrophic activities in the mouse. The relative affinities of the analogues for the receptors in the uterine tissue was similar to those determined for the receptor in solution. The SH and $NHSO_2CH_3$ analogues were without any activity in all the tests. Two analogues, with H or OCH_3 disubstitution were uterotrophic without having receptor affinities. The other analogues (except the *p*,*p*'-NH_2-analogue) were markedly less uterotrophic than expected from their relative receptor affinities. For example, the *p*-OH,*p*'-NH_2-analogue had 85 % of the receptor affinity of *meso*-hexoestrol but only 0.5 % of the uterotrophic potency. On the other hand, when this analogue or *meso*-hexoestrol was *injected*, it was found that the analogue had much less competitive affinity for the uterine receptors and was less efficiently retained than *meso*-hexoestrol. No compound was anti-uterotrophic.

Key-words. Hexoestrol – oestrogens – oestrogen receptors – uterus hydrogen-bonding.

In an accompaning communication (TERENIUS 1972) the binding affinities are described for a series of p p'-substituted analogues of the synthetic oestrogen *meso*-hexoestrol for the oestrogen receptors in the mouse uterus. These data showed that hydrogen bonding to the receptor via at least one hydrogen bond is essential.

In the present communication, the tissue affinities of the analogues were tested in order to find out whether the receptors *in situ* (the mouse uterus) had the same binding specificity as the receptors in a cell-free system. In addition, uterotrophic activities as well as anti-uterotrophic activities of the analogues were measured.

Materials and methods

Test compounds are described in the previous paper (TERENIUS 1972).

Animals. Immature female mice of the NMRI strain were used. They weighed 8.5–9 g at the start of the experiments. They were kept in uniform conditions with commercial mouse cubes and tap water available without restriction.

Uterotrophic activity was assayed by a Rubin test. The animals were given 0.1 ml of a solution of the test compound in olive oil subcutaneously each day for 3 days. Autopsy was performed 24 hours after the final injection. At autopsy body weights were obtained and the uteri were cleaned of adherent tissues, blotted and dried at 100° for at least 24 hours before weighing.

Anti-uterotrophic activity was assayed by a similar injection schedule. The doses of a potential antagonist were injected simultaneously with 0.015 µg oestradiol-17β-3-benzoate. *Both the antagonist and the oestrogen were injected subcutaneously but at* different sites in 0.05 ml olive oil. The uterine dry weights from 8 animals per group were obtained as above.

In vitro experiments were performed as previously described (TERENIUS 1971). Uteri were incubated for 1 hour at 37° in flasks with a fixed concentration of labeled oestradiol 17β (3.6×10^{-9} M) and varying concentrations of test substances. The uteri were then subjected to washing for 1 hour at 25° in a buffer with 3.6×10^{-7} M non-labeled oestradiol-17β. At each concentration uteri from at least 4 mice were incubated. The experiments included control flasks in each run and the competitive binding activity was expressed in percent of the control (Fig. 1). These measurements were performed under conditions where the receptor sites were not saturated.

Fig. 1. Effect of hexoestrol analogues on the binding of oestradiol-17β to mouse uterus *in vitro*. Each point is the mean of 4 to 6 samples. The S. E. M. of all values were within ± 10 % of the means.

The tissues were digested with Soluene and scintillation solution was added. The counts recorded on a liquid scintillation counter were transformed to disintegrations per min. (d.p.m.).

Results

The hexoestrol analogues were tested for their ability to inhibit the binding of labelled oestradiol 17β to the uterus (fig. 1). It was found that the analogues could be distributed into 4 groups with regard to their competitive binding affinity. The OH, NH_2-analogue was almost as potent as *meso*-hexoestrol (R=R' =OH); the OH, H, the OH, CH_2OH, the OH, OCH_3 and the OH, F analogues were at least 10 times less potent while the NH_2, NH_2, the OH, COOH and the CH_2OH, CH_2OH analogues were 20–100 times less potent. Finally, several compounds were found to be devoid of activity at the highest tested concentration, 5×10^{-5}M. This was also true for the SH, SH analogue. Since thiol groups are oxidizable structures a competitive experiment was run in the presence of 10^{-3}M of the reducing agent dithioerythritol. The presence of this agent did not influence the results.

The uterotrophic potencies of the compounds are shown in fig. 2. *meso*-Hexoestrol was by far the most active compound. The OH, CH_2OH and the OH, COOH analogues gave anomalous (flat) dose – effect curves. These compounds as well as the NH_2, NH_2 analogue did not seem to produce full responses. The compounds listed as inactive produced no response at a daily dose of 300 μg per animal.

Anti-uterotrophic activity was assayed for the following analogues (daily doses): CH_2OH, CH_2OH (10, 100 μg), NH_2, NH_2 (30, 100 μg), OH, CH_2OH (0.5, 5 μg), OH, COOH (1.5, 15 μg) and OH, NH_2 (0.015, 0.05, 0.15 μg). None of the compounds showed any significant anti-uterotrophic activity.

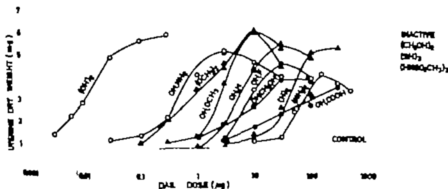


Fig. 2. Uterotrophic activity (increase in mg dry uterus/10 g body weight) in relation to logarithmic dose (μg/mouse/day). 5 animals per group.

Table 1

Competitive binding affinities for *meso*-hexoestrol and its p'-NH_2 analogue for the mouse uterus *in vivo*

A. Hexoestrol or its analogue (1 μg of each) in 10 % propylene glycol in saline or in olive oil was injected at various times before the intravenous injection of 0.01 μg of oestradiol 17β-^{3}H in saline. The animals were killed 1 hour after the injection of oestradiol-^{3}H.

B Hexoestrol or its analogue (1 μg of each) was injected together with 0.01 μg of oestradiol-17β-^{3}H.

All injection volumes were 0.1 ml. Mean values and S. E. M. are given for 7 animals per group.

A

Compound R	R	Vehicle/Injection	Time interval between injections	D.p.m./mg wet weight
OH	OH	Saline/Intravenous	30 min	227 ± 20
OH	NH_2			837 ± 61
OH	OH	Saline/Intravenous	6 hours	1013 ± 72
OH	NH_2			2132 ± 100
OH	OH	Olive oil/ Subcutaneous	3 hours	303 ± 38
OH	NH_2			1822 ± 168
None (Vehicle)		Saline/Intravenous	30 min	1166 ± 64

B

Compound R	R	Vehicle/Injection	D p.m./mg wet weight
OH	OH	Saline/Intravenous	333 ± 13
OH	NH_2		672 ± 65
None		Saline/Intravenous	1141 ± 88

Since the hexoestrol analogues were unexpectedly weak in the uterus-growth test, one of them, the OH, NH_2-analogue, was compared with hexoestrol for competitive receptor binding *in vivo* (table 1). Hexoestrol was much more active as an inhibitor than the analogue which had a weak inhibitory effect when given in connection with oestradiol-17β. When pre-injected several hours before oestradiol 17β, the analogue actually increased the binding of oestradiol-17β over the controls ($P < 0.001$ in Student's t-test).

A comparison between the binding affinities to receptors in the cell-free system (TERENIUS 1972), the binding capacities to uterine tissue *in vitro* and the uterotrophic activities is shown in table 2. The uterotrophic potencies of the OH, CH_2OH and the OH, COOH analogues relative to the others are difficult to estimate because of the non-parallel dose response curves, an arbitrary response level, i.e. an increase from 1 to 3 mg, was taken as refer ence in order to get some information.

Discussion

Several of the compounds studied are known in the literature but except for *meso*-hexoestrol and its methyl-ether very few biological data are available. The OH, NH_2 analogue has been reported to possess about 1 / of the uterotrophic potency of hexoestrol in rats (BIGGERSTAFF & WILDS 1949)

Table 2

Receptor affinities (R), tissue affinities (T) and oestrogenic activities (E) of hexoestrol analogues.

R-⟨O⟩-CH(C_2H_5)-CH(C_2H_5)-⟨O⟩-R		Equiactive molar ratios				
R	R	R[1]	T[2]	E[3]	T/R	E/R
OH	OH	100	100	100	1	1
OH	NH_2	85	66	0.5	0.78	0.0059
OH	CH_2OH	63	10	0.083	0.16	0.0013
OH	OCH_3	67	8.3	0.56	0.12	0.0083
OH	F	30	7.7	0.14	0.25	0.0047
OH	H	12	13	0.22	1.1	0.018
OH	COOH	0.85	1.0	0.01	1.2	0.012
CH_2OH	CH_2OH	0.018	0.66	–	36	–
NH_2	NH_2	0.085	4.0	0.13	47	1.5
H	H	–		0.083	–	–
OCH_3	OCH_3			1.7	–	–
SH	SH		–	–	–	–
$NHSO_2CH_3$	$NHSO_2CH_3$	–		–	–	–

[1] K_i values (TERENIUS 1972)
[2] Endpoint. 50 % inhibition (fig. 1).
[3] Endpoint. 3 mg uterine dry weight (fig. 2).

while an isomer mixture of the OH COOH analogue was less active (NEHER & MIESCHER 1946) These results in rats are similar to the present findings in mice.

It was found that measurements of binding affinities in the cell free system or with tissue gave similar results for all active compounds and both tests showed 4 compounds to be inactive. This shows that the receptors *in situ* and in solution have similar binding properties, and furthermore that tissular or cellular barriers are not very important for accumulation. However for 2 compounds with low binding potencies (the NH_2, NH_2 and CH_2OH, CH_2OH analogues) the cell-free system gave much (40 times) lower relative affinities. The reason for this may lie in the non-specific effects occurring at the elevated concentrations which it was necessary to use.

When uterotrophic activities were compared with the affinities obtained in either test a poor correlation was obtained (table 2) Roughly speaking, the relative potency of hexoestrol *in vivo* was 50 to 100 times higher than expected from the *in vitro* results. Two compounds, the di-OCH_3 and di-H analogues were somewhat uterotrophic without being active *in vitro* It is reasonable to assume that this is due to hydrolysis and hydroxylation respectively giving one or two free *p*-hydroxyls.

The discrepancy between binding affinities and uterotrophic activities might be taken as an argument against the concept that the oestrogen binders are the receptors. However the experiments in table 1 clearly show that *in vivo* the competitive affinity of the OH, NH_2 analogue is much lower than expected from the *in vitro* results. Furthermore, the OH, H analogue has about 2 / the competitive affinity of hexoestrol when given in oil 3 hours before oestradiol-^{3}H as in table 1 (TERENIUS 1966) These *in vivo* estimates of affinity are thus in much better agreement with activity *in vivo*

Why is it that *in vitro* affinities are so high in comparison with *in vivo* affinities? The explanation may of course lie in differences in pharmacokinetics *in vivo* i.e., in the amounts which actually reach the uterine tissue. Another possibility could be differences in the dynamics of the oestrogen-receptor complexes. Potent oestrogens like *meso*-hexoestrol and oestradiol-17β are not only bound effectively but the receptor complexes are also stabilized in some way Previous studies by the author have emphasized this point (TERENIUS 1971). The monohydroxy hexoestrol analogues may well occupy the receptor population for shorter periods of time than hexoestrol itself. A weak inhibition was also observed when the OH NH_2 analogue was given in connection with oestradiol 17β but this was not so when the injection interval was increased (table 1). Actually the OH, NH_2 analogue significantly increased the oestradiol-17β uptake after longer pre-injection periods, a phenomenon also known to occur after the injection of the OH, H analogue (TERENIUS 1966) and oestradiol (KRAAY & BLACK 1970). The situation is obviously complicated

and the opinion held by KORENMAN (1969) that binding affinities are synonymous with oestrogenic activity is an over simplification.

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From the Department of Histology Karolinska Institutet,
S-104 01 Stockholm, Sweden

Noradrenaline Uptake Mechanisms in Human Atrium

By

Charlotte Sachs and Gösta Jonsson

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Abstract The ^{3}H-amine uptake and accumulation *i vitro* in human and rat heart tissue was compared. The equilibration and clearance of ^{3}H-sorbitol was the same in both species, indicating similar diffusion conditions in the extracellular space. The uptake of ^{3}H-metaraminol (^{3}H-MA) in non-nervous tissue was measured by incubation at 0 or preincubation in desipramine, both procedures known to inhibit effectively the axonal 'membrane pump uptake mechanism. The extraneuronal uptake in man and rat thus measured was similarly low A high correlation was found between neuronal ^{3}H MA uptake and endogenous noradrenaline (NA) concentration measured in the same human atria, thus indicating the possibility of quantitatively estimating nerve density by measuring ^{3}H-amine uptake. The neuronal uptake process for ^{3}H-MA in heart tissue from both man and rat was found to obey Michaelis-Menten kinetics, whereas the affinity for the uptake sites was somewhat higher in the rat. Reserpine caused a reduced accumulation of ^{3}H NA in both species, which could be counteracted by the monoamine oxidase inhibitor nialamide. The spontaneous release of ^{3}H NA previously taken up and accumulated was increased by reserpine in heart tissue of both man and rat. Pre-incubation in 6-hydroxydopamine led to a reduced uptake of ^{3}H MA, which was more pronounced in rat heart slices. The effect of certain drugs, known to affect monoamine uptake-storage mechanisms, on the ^{3}H-MA uptake in heart slices from man and rat were studied. All the drugs investigated displayed a concentration dependent inhibition of the ^{3}H MA uptake. The order of inhibition potency was desipramine > protriptyline > imipramine > chlorpromazine > chlorimipramine. All the drugs studied induced a more marked uptake inhibition in human than in rat heart tissue. Th adrenergic nerves of human and rat heart thus showed qualitative similarities regarding uptake and storage mechanisms, but some quantitative differences were found, e. g. a higher resistance to the neurotoxic ffect of 6-hydroxydopamine and a more easily blocked 'membrane pump by certain uptake blocking drugs in the human heart. It is suggested that these differences might be due to a less efficient 'membrane pump in human adrenergic nerves.

Key-words: 6-hydroxydopamine – noradrenaline – desipramine – chlorimipramine – *imipramine – chlorpromazine.*

Uptake mechanisms of catecholamines have received a good deal of attention to during the last decade, especially those in adrenergic nerves, since they are considered to play an important role in the inactivation of both circulating and released catecholamines (AXELROD *et al.* 1959 WHITBY *et al.* 1961). The adrenergic nerves possess two main uptake and accumulation mechanisms, one located to the axonal membrane of the whole adrenergic neurone, the so called 'membrane pump' and one to the intraneuronal amine storage granules. In addition to the neuronal uptake mechanisms, uptake of noradrenaline has also been found to occur at certain extraneuronal sites, such as the walls of arteries (GILLESPIE 1968), heart muscle cells (SACHS 1970) and special connective tissue cells in the rat heart (FARNEBO & MALMFORS 1969) (The following abbreviations will be used. NA = noradrenaline MA = metaraminol 6-OH DA = 6-hydroxydopamine· MAO = monoamine oxidase DMI = desipramine).

Although a great deal of information concerning NA uptake mechanisms has accumulated during the last decade, practically all this information derives from studies on experimental animals, and very little is known at present about these mechanisms in human tissues. A brief study on this subject has been published recently (SACHS 1969) where it was found that the human atrial appendage was richly innervated by adrenergic nerves, the distribution and appearance of which were similar to that of other species, e. g. mouse and rat. It was further shown that the adrenergic nerves of human atria possess an efficient uptake and accumulation mechanism for NA. The purpose of the present investigation was to characterize the NA uptake processes in human heart tissue in more detail and to compare the effects of certain drugs of clinical importance, which are known to influence neuronal NA uptake mechanisms, on the ^{3}H NA uptake in human and rat heart tissue.

Material and Methods

Right atrial appendages, which would otherwise have been discarded, was obtained from 28 patients undergoing heart operations. The age varied from 18 to 62 years. The main diagnoses were: aortic incompetence (8) atrial septal defect (5), ventricular septal defect (3), aortic stenosis (1) mitral stenosis (1), mitral incompetence (1), tetralogy of Fallot (1), and combined valvular defects (8). Five patients had signs of heart decompensation at rest, when examined preoperatively The medical treatment was digoxin® (0.25 mg × 1 n = 18), Librium® (diazepam, 5 mg × 3, n = 2). The patients were premedicated before the operation with morphine and scopolamine.

The heart tissue was transported in cold saline and transferred within 15 min. to cold Krebs-Ringer bicarbonate buffer of pH 7.4 Slices with a thickness of about 0.5 mm were immediately prepared from human atrial appendages and from rat heart ventricles (male Sprague-Dawley 175 g). The slices were, if not otherwise stated incubated at 37° for 10 min. in a Krebs-Ringer bicarbonate buffer (see H[illegible]

1967) containing 10^{-7}M ^{3}H-NA or ^{3}H-MA. After the incubation the tissue was rinsed in cold buffer for a few seconds, carefully blotted on filter paper and weighed on a Cahn electrobalance and thereafter dissolved in 0.5 ml Soluene™-100 (Packard Instr. Co. Inc.). Ten ml of toluene scintillation solution was added and the radioactivity determined in a liquid scintillation spectrometer (Packard Tri-Carb Model 3320). In some experiments, when a considerable amount of ^{3}H-NA taken up and accumulated is known to be metabolized, ^{3}H-NA was isolated and determined by specific chemical analytical procedures (CARLSSON & WALDECK 1963, for details see SACHS 1969). The values were expressed as d.p.m./mg heart tissue. Quenching was monitored by addition of a standard amount of ^{3}H-toluene and was found to be consistent throughout the study. Counting efficiency: 25 %. In most experiments only total radioactivity was measured, since the main part, about 90 %, has previously been shown to constitute unchanged ^{3}H-NA (SACHS 1969). ^{3}H-MA does not undergo metabolic degradation (see LUNDBORG 1967), thus making it possible to measure ^{3}H-MA uptake by determination of total radioactivity.

Endogenous NA was determined according to the method of BERTLER *et al.* (1958). *MAO determination* was made according to WURTMAN & AXELROD (1963).

The following substances were used: *dl*-noradrenaline-7-^{3}H-HCl (5–9 ci/mmol), tryptamine 2-^{14}C-bisuccinate (10.9 mci/mmol), *dl*-metaraminol 7-^{3}H-HCl (16 ci/mmol, New England Nuclear Corp., USA), ^{3}H-sorbitol (1.43 ci/mmol, The Radiochemical Center, Amersham, England), reserpine phosphate, dissolved in 5.5 % glucose (Swedish Ciba), nialamide-HCl (niamid®, Pfizer), 6-hydroxydopamine-HCl (H88/32, Hässle), chlorpromazine-HCl (hibernal®, Leo), imipramine-HCl (tofranil®, Geigy), desipramine-HCl (pertofran®, Geigy), chlorimipramine-HCl (Geigy), protriptyline-HCl (Merck, Sharp & Dohme).

Results

Sorbitol space. The sorbitol space was determined by incubating heart slices from man and rat in buffer containing ^{3}H-sorbitol. There was rapid tissue equilibration which seemed to be complete within 10 min. (fig. 1). The same results were obtained for both species. The sorbitol space =

$$\frac{\text{d.p.m. } ^3\text{H-sorbitol/mg tissue}}{\text{d.p.m. } ^3\text{H-sorbitol/}\mu\text{l medium}} \times 100$$

was calculated to be about 25 % both in the human and rat heart tissue. Clearance of ^{3}H-sorbitol from the tissue was equally rapid in man and rat (fig. 1). In five min. practically all of the extracellular volume so estimated, was cleared.

Uptake of ^{3}H-MA. The time-course of ^{3}H-MA uptake was studied in rat heart slices (fig. 2). There was a linear uptake up to 30 min. of incubation in 10^{-7}M ^{3}H-MA, while with 10^{-6}M ^{3}H-MA, there was a deviation from linearity between 5 and 10 min. of incubation (*cf.* SACHS 1969).

In order to obtain information as to the amount of ^{3}H-MA (10^{-7}M or 10^{-6}M, 10 min.) taken up in non-nervous tissue, (= extraneuronal uptake) heart slices were incubated at 0° or preincubated in 10^{-5}M DMI (table 1). Both these procedures are known to block effectively the uptake in the adrenergic nerves (see JONSSON *et al.* 1969, SACHS 1970). The extraneuronal

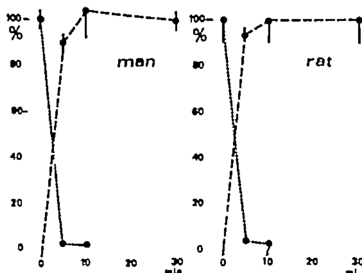


Fig. 1. Time-course of ^{3}H-sorbitol (2.5 μci/ml) equilibration *in vitro* in heart slices from man and rat (● -- ●). The values are expressed as percentages of the 30 min. value. Time-course of ^{3}H-sorbitol clearance in heart slices previously incubated in ^{3}H-sorbitol (2.5 μci/ml) for 30 min. (● ●). The tissue was then transferred to fresh buffer and reincubated for 5 or 10 min. The values are expressed as percentages of the zero time value. Each point represents the mean ± S. E. M. of 4 determinations.

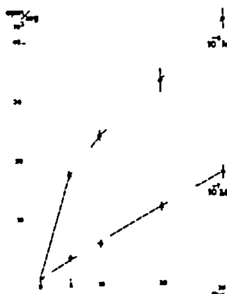


Fig. 2. Time-course of ^{3}H MA uptake and accumulation *in vitro* in rat heart slices. The tissue was incubated in ^{3}H-MA (10^{-7} and 10^{-6}M) for 5, 10, 20 and 30 min. Each point represents the mean ± S.E.M. of 4 determinations.

Table 1

Effect of DMI and 0 on the ^{3}H MA uptake in heart tissue from man and rat.

^{3}H MA conc.	MAN 0	MAN DMI	RAT 0	RAT DMI
10^{-6}M	16 ± 2.4 (15)	15 ± 1.7 (13)	17 ± 1.9 (10)	18 ± 1.6 (16)
10^{-7}M	7 ± 1.1 (15)	5 ± 0.6 (14)	10 ± 0.7 (15)	6 ± 0.8 (16)

The heart slices were either preincubated in DMI (10^{-6}M, 10 min.) and then transferred to ^{3}H MA (10^{-7}M or 10^{-6}M, 10 min.) or incubated in ^{3}H MA (10^{-7}M or 10^{-6}M, 10 min.) at 0 The values are expressed as percentages of the control (^{3}H-MA 10^{-7}M or 10^{-6}M, 10 min., 37°). Number of determinations in brackets.

uptake of ^{3}H MA so evaluated, was higher with the higher concentration of ^{3}H MA and was similar in the human and rat heart tissue and for the two ways of making the estimations.

NA content in human heart The endogenous NA content varied considerably in the human heart biopsies, in all probability due to the variation in density of adrenergic nerves in different parts of the cut out atrial appendages (*cf* SACHS 1969). The mean content was. 1 15 µg NA per g heart tissue (S.E.M. ± 0.38 n.11), which is approximately the same as that previously published by CHIDSEY *et al.* (1963). Rat heart is known to contain about 1 µg/g NA (see e. g. IVERSEN 1967)

There was a high positive correlation ($r = 0.91$,) between neuronal ^{3}H MA uptake, d.p.m./mg, (10^{-7}M, 10 mm. at 37 with the ^{3}H MA uptake at 0° subtracted) and the endogenous concentration, µg/g, in human heart tissue. Slices from one heart were incubated in ^{3}H MA and the remainder of the biopsy was taken for chemical analytical determination of NA. These results show that it is possible to obtain information about the nerve density by measuring ^{3}H MA uptake.

Michaelis-Menten kinetics Reciprocal analysis of the neuronal uptake, process (extraneuronal uptake measured as uptake at 0° was subtracted) for ^{3}H-MA (10^{-7}M) during a 10 min. incubation, in heart slices from man and rat and plotted according to LINEWEAVER & BURK (1934), revealed that the neuronal uptake process in both species obeyed Michaelis-Menten kinetics (fig. 3)

Retention of ^{3}H-NA taken up Retention of ^{3}H NA was investigated by first incubating slices from human and rat hearts in 10^{-7}M ^{3}H NA for 30 min. and then re-incubating the slices for 10 30 or 60 min. in isotope-free buffer There was a rapid disappearance of the ^{3}H-NA previously taken

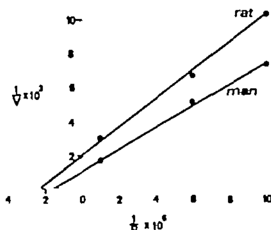


Fig. 3. Neuronal ^{3}H MA uptake in vitro in heart slices from man and rat, plotted according to LINEWEAVER & BURK (1934). The incubation time was 10 min. The values are corrected for radioactivity present extraneuronally by subtracting the uptake values at 0 . Each point is the mean of 8–12 determinations. The lines were calculated by linear regression analysis. $r_{man} = 0.99$, $r_{rat} = 1.00$, $Km_{man} = 0.70 \times 10^{-6}$M, $Km_{rat} = 0.57 \times 10^{-6}$M, $Vmax_{man} = 877$, $Vmax_{rat} = 457$. Vmax is expressed as ng/g/10 min. Velocity (V) = uptake of ^{3}H MA cpm/mg/10 min. Substrate concentration (S) = M ^{3}H-MA.

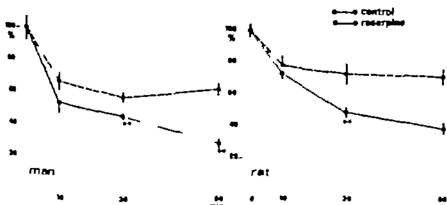


Fig. 4. Effect of reserpine (10^{-4}M) on the disappearance of ^{3}H-NA previously taken up in vitro (10^{-7}M, 30 min.) in heart slices from man and rat. The slices after the incubation in ^{3}H NA were transferred to isotope-free buffer with or without reserpine and reincubated for 10, 30 or 60 min. The values are expressed as percentages of the zero time value. Each point represents the mean ± S.E.M. of 4 determinations. The difference in values between reserpine and the respective control incubation was tested with student's t-test. ** $0.01 > p > 0.001$, $P < 0.001$.

up during the first 10 min. of reincubation (fig. 4), after which the curve levelled off. When reserpine (10^{-6}M) was added to the reincubation medium, there was a more rapid loss of ^{3}H NA. The disappearance was somewhat more pronounced in slices from the human heart as compared with those from the rat heart.

Effect of reserpine and nialamide on ^{3}H-NA uptake. Human heart slices were first preincubated in reserpine (10^{-6}M) or reserpine (10^{-6}M)+ nialamide (50 μg/ml) for 60 min. before the incubation in ^{3}H-NA (10^{-7} or 10^{-6}M, 30 min. fig. 5). The ^{3}H NA taken up and accumulated was isolated and specifically determined. Preincubation in reserpine resulted in a marked reduction in the uptake and retention of ^{3}H NA, which was partially counteracted by nialamide.

Incubating human heart slices in nialamide (50 μg/ml) for 60 min. caused only a 65 % inhibition of MAO (determined according to WURTMAN & AXELROD 1963) compared with tissue incubated for the same period of time in buffer only.

Effect of certain drugs on ^{3}H MA uptake. The effect of preincubating slices from human and rat hearts in 6-OH DA (10^{-4} or 10^{-3}M) for 30 or 60 min. on the ^{3}H MA uptake was studied (fig. 6). There was a reduction in

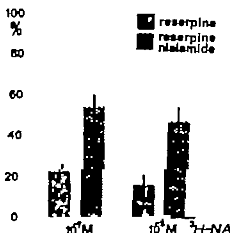


Fig. 5. Effect of reserpine and nialamide on the uptake of ^{3}H-NA *in vitro* in human heart slices. The tissue was preincubated in buffer containing reserpine (10^{-6}M) or reserpine (10^{-6}M) + nialamide (50 μg/ml) for 60 min., thereafter transferred to buffer containing ^{3}H-NA (10^{-7} or 10^{-6}M) and incubated for another 30 min. The control tissue was preincubated in buffer for 60 min., before the incubation in ^{3}H-NA. The values are expressed as percentages of the control (100 ± 5–8 %). ^{3}H-NA was specifically chemically determined. Each column represents the mean ± S.E.M. of 4 determinations.

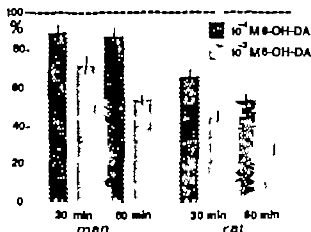


Fig. 6. Effect of 6-OH DA on the uptake of ^{3}H MA *in vitro*. Heart slices from man and rat were preincubated in buffer containing 6-OH-DA (10^{-4} or 10^{-3}M) for 30 or 60 min., thereafter rinsed for 5 min. in fresh buffer and then incubated in ^{3}H MA (10^{-7}M) for 10 min. The control tissue was preincubated in buffer for 30 or 60 min. The values are expressed as percentages of the control. Each column represents the mean ± S.E.M. of 8–16 determinations. The difference in values between man and rats was tested with student's t-test. $0.01 > P > 0.001$.

^{3}H-MA uptake which was dose and time related to the preincubation in 6-OH-DA. The decrease in uptake was more marked in rat heart slices.

The effect of some psychoactive drugs, known to affect neuronal uptake mechanisms, were compared with the ^{3}H-MA uptake in human and rat heart tissue (fig. 7). The slices were incubated with the respective drug (10^{-6} or 10^{-5}M) together with ^{3}H-MA (10^{-7}M) for 10 min. All the drugs studied induced a marked decrease in the uptake of ^{3}H MA. The uptake inhibition was concentration dependent and in all cases more pronounced in human than in rat heart tissue. The order of uptake inhibition potency was, DMI > protriptyline > imipramine > chlorpromazine > chlorimipramine.

Discussion

Previous fluorescence histochemical studies have shown that the NA present in the heart of e. g. rat, mouse and man is almost exclusively confined to the post-ganglionic sympathetic adrenergic nerves (Jacobowitz 1967, Jonsson & Sachs 1969, Sachs 1969). The characteristic varicose nerve terminals have the same appearance and distribution in the human atrium as in other mammalian species (Sachs 1969, McLean 1968) which agrees well with the morphology of the autonomic ground-plexus in rat atrium visualized by means of methylene blue staining (Hillarp 1959).

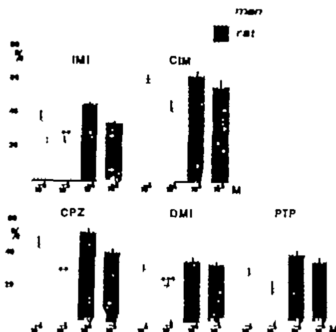


Fig. 7 Effects of certain drugs on ^{3}H MA uptake *in vitro*. Human and rat heart slices were incubated with 10^{-6} or 10^{-5}M of the drug studied, together with ^{3}H-MA (10^{-7}M) for 10 min. The values are expressed as percentages of the control (100 ± 5–9 %). Each column represents the mean ± S.E.M. of 4–8 determinations. CPZ = chlorpromazine, DMI = desipramine, PTP = protriptyline, IMI = imipramine and CIM = chlorimipramine. The difference in values between man and rat was tested with student's t-test.

$0.05 > P > 0.01$ ** $0.01 > P > 0.001$ ** $P < 0.001$.

In order to be able to make proper comparisons and adequate interpretations of *in vitro* uptake data obtained from different tissues, it is important that the equilibration conditions for the extracellular space are similar for the different tissues. The present results obtained with ^{3}H-sorbitol, which is a marker for the extracellular space (see MORGAN *et al.* 1961) clearly show that the diffusion conditions were the same for slices from both human and rat heart. Thus it was found that ^{3}H-sorbitol promptly equilibrated in and was rapidly cleared from both types of slices. *The ^{3}H-sorbitol space* (∼ extracellular space) was calculated to be about 25 / both in human and rat heart slices, which is approximately the same as that found in the perfused rat heart (IVERSEN 1965) and in mouse atrial appendage incubated *in vitro* (SACHS 1970).

The adrenergic nerve terminals show a varying distribution with a denser network in the atria than in the ventricles both in the rat and mouse heart, which is reflected in a higher endogenous NA concentration in the atria

(MUSCHOLL 1959; ANGELAKOS *et al.* 1963; SACHS 1970). The density of the nerve-plexus can also vary to a fairly great extent within the various parts of the heart. In the present study the ³H MA uptake (d.p.m./mg) varied considerably from patient to patient, while the uptake was constant in rat heart slices. It was therefore thought of interest to compare the endogenous NA content with the ³H MA uptake in slices from the same human atrium. There was a good correlation between these two parameters, which is in agreement with previously reported results (WURTMAN *et al.* 1964; KOPIN *et al.* 1965). A close correlation between *nerve* density and ³H-amine uptake, e. g. ³H MA or ³H-NA, has also been found (OLSON & MALMFORS 1970; JONSSON & SACHS 1972). In view of this, it is fairly easy to obtain a quantitation of the nerve density by measuring ³H-MA uptake. This might be of special value when studying human tissues, where it is not always possible to obtain tissue from exactly the same region of an organ. The variations in ³H MA uptake seen in atrial tissue slices from one patient to another are in all probability related to variations in density of the adrenergic innervation, although small effects of the different therapeutic medications and/or changes secondary to the diseases cannot be excluded. It has, however, been reported that the NA content in human atria is not influenced by medical therapy, age or diagnosis (CHIDSEY *et al.* 1963). Furthermore, none of the drugs used by some of the patients (see Material and Methods) are known to be potent uptake blockers. The present results do not allow any conclusion as to any changes in ³H MA uptake properties related to any special heart disease.

MA has been found to be useful for studies on uptake mechanisms of adrenergic nerves, since this compound is rapidly taken up by the axonal membrane pump and incorporated in the intraneuronally located amine storage granules (see LUNDBORG 1967). In addition MA is not catabolized by MAO or catechol-O-methyl transferase, which makes it easy to investigate uptake mechanisms with ³H MA by measuring only total radioactivity.

The ³H-MA uptake was reduced to very low and similar values for slices from both human and rat tissue when the incubation was performed at 0° which certainly is related to an inhibition of the energy-yielding metabolic processes necessary for the membrane pump mechanism (HAMBERGER 1967; JONSSON *et al.* 1969; SACHS 1970). Equally low ³H MA uptake values were obtained when pre-incubating in 10^{-5}M DMI, which in this concentration is known to block completely the membrane pump (see HAMBERGER 1967). The ³H-MA uptake during these conditions must be considered to be extraneuronally located. After the incubation of human atrial slices in a high concentration of NA, it has been found that the extraneuronal uptake is not localized to any specific structures, but evenly distributed in the muscle cells (SACHS 1969) as also found in the mouse atrium (SACHS 1970). There has

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From the Department of Pharmacology University of Bergen,
MFH Bygget, 5000 Bergen, Norway

Metabolism of Xenobiotics by Strains of Intestinal Bacteria

By

Harald A. Soleim and Ronald R. Scheline

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Abstract: The metabolism of some xenobiotic compounds when incubated with strains of intestinal bacteria has been studied. The reactions included: 1) glucuronide hydrolysis, 2) glucoside hydrolysis, 3) sulphate ester hydrolysis, 4) glycine conjugate hydrolysis, 5) N-acetyl derivative hydrolysis, 6) amide hydrolysis, 7) ester hydrolysis, 8) dehydroxylation, 9) decarboxylation 10) double bond reduction, 11) nitro reduction, 12) azo reduction and 13) heterocyclic ring scission. The results showed that strains of the following test microorganisms were able to carry out the indicated reactions to varying degrees. *Str faecalis* (4, 11, 12), *E. coli* (1, 11, 12), *Lactobacillus* (2, 10, 11 12) *Bacillus* (9 11 12), *Ps. aeruginosa* (12), *Pr vulgaris* (2, 10, 11 12), *A aerogenes* (2, 3, 4 5, 9 11 12, 13), *Clostridium* (10, 11, 12) *and Bacteroides* (1, 11 12). These results are discussed in relation to earlier findings on the metabolism of xenobiotics by members of the intestinal microflora.

Key-words: Metabolism bacteria.

There has recently developed an increasing awareness of the fact that xenobiotic compounds may undergo metabolism by the intestinal microflora (SCHELINE 1968d WALKER 1970 DRASAR *et al* 1970 WILLIAMS 1970; WILLIAMS 1971 SCHELINE 1972) Hitherto most of the relevant investigations have used mixed cultures of intestinal bacteria It would obviously be of considerable interest to ascertain which intestinal microorganisms are responsible for the metabolic transformations found to occur with particular xenobiotics. Such information could be especially useful in correlating individual or species differences in metabolism with the variations in the intestinal microbial population which are known to exist (DRASAR *et al* 1970) The present investigation was therefore undertaken with the aim of associating particular reactions with particular microorganisms. For this pur

pose a number of xenobiotic compounds sufficient to furnish a representative range of reactions were incubated with isolated strains of some common intestinal bacteria.

Methods

Compounds.

All compounds, except 4-hydroxylaminobenzoic acid which was prepared by the method of BAUER & ROSENTHAL (1944), were either obtained from commercial sources or were available in this laboratory from earlier investigations.

Isolation of bacteria.

Standard microbiological methods were used (COLLINS 1964 THE OXOID MANUAL 1965) The following bacteria were obtained from the caecal contents of an inbred strain of albino rats maintained on a commercial pellet diet (Felleskjøpet, Oslo): *Escherichia coli, Streptococcus faecalis var liquefaciens Lactobacillus* sp. nr 1, *Bacillus* sp. nr 3 and *Clostridium* sp. *Bacillus* sp. nr 1 was available from an earlier study (INDAHL & SCHELINE 1968) Unsuccessful attempts were made to isolate *Bacteroides*,

Metabolism of xenol

Compound	Reaction	*Streptococcus faecalis*
4-Methylumbelliferone glucuronide	Hydrolysis of glucuronide	0[1] (2)[2]
Phenyl-β-D-glucoside	Hydrolysis of glucoside	0 (2)
4-Nitrocatechol sulphate	Hydrolysis of sulphate ester	0 (2)
4-Aminohippuric acid	Hydrolysis of glycine conjugate	++ (4)
4-Acetamidobenzoic acid	Hydrolysis of N-acetyl derivative	0 (2)
N[1]-Acetylsulphanilamide	Hydrolysis of N-acetyl derivative	0 (2)
Succinylsulphathiazol	Hydrolysis of amide	0 (2)
Methyl gallate	Hydrolysis of ester	0 (4)
3,4-Dihydroxyphenylpropionic acid	Dehydroxylation	0 (2)
4-Hydroxybenzoic acid	Decarboxylation	0 (2)
3-Hydroxycinnamic acid	Reduction of double bond	0 (2)
4-Nitrobenzoic acid	Reduction of nitro group[3]	++ (2)
Methyl red	Reduction of azo group	+++ (2)
Acid yellow	Reduction of azo group	+++ (3)
Coumarin	Scission of heterocyclic ring	0 (2)

1 Symbols: 0, none, + minor ++ moderate; +++ extensive.

2 Number in brackets refers to number of experiments.

3 See text.

Aerobacter aerogenes Proteus vulgaris and *Pseudomonas* from rat caecal contents. The remaining microorganisms listed in table 1 were therefore obtained from The Gade Institute, Department of Microbiology University of Bergen, School of Medicine through the generosity of Professor P Oeding.

Incubation with bacteria.

The general method used in these experiments consisted of incubation of the test compound (2.5–10 mg) in a liquid incubation medium (10 ml) for 18 hours at 37° with or without a suspension (1 ml) of the particular microorganism. In addition, a blank consisting of the medium and the microorganism was incubated. The medium usually used contained glucose, yeast extract and peptone in 0.1 M phosphate buffer (pH 7.4) as described previously (SCHELINE 1968a). The MRS-medium of MAN *et al.* (1960) was used with *Lactobacillus* and AC-medium (DIFCO MANUAL 1953) was used with *Clostridium*. The incubation medium used with *Bacteroides* was Brain Heart Infusion (Difco) in phosphate buffer (pH 7.4) containing agar (0.25 %). The incubation tubes were fitted with cotton plugs except when *Clostridium* and *Bacteroides* were incubated. Here, tightly stoppered tubes nearly full of medium were used. The incubation time was increased to approximately 48 hours with *Bacteroides*. The incubates containing 4-nitrocatechol sulphate employed a buffer of pH 6.8 to prevent the spontaneous hydrolysis which occurred with the usual buffer

...stinal bacteria.

...to-...llus ...r.1	*Lactobacillus* sp. nr 2	*Bacillus* sp. nr 1	*Bacillus* sp. nr 3	*Pseudomonas aeruginosa* sp. nr 1	*Pseudomonas aeruginosa* sp. nr 2	*Proteus vulgaris*	*Aerobacter aerogenes*	*Clostridium* sp.	*Bacteroides* sp.
0 (3)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (4)	0 (2)	[illegible] (2)
0 (3)	[illegible] (4)	0 (2)	0 (2)	0 (2)	0 (2)	[illegible] (3)	[illegible] (4)	0 (2)	0 (2)
0 (2)	0 (2)	0 (3)	0 (2)	0 (3)	0 (3)	0 (3)	+ (3)	0 (4)	0 (2)
0 (3)	0 (3)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	++ (3)	0 (2)	0 (2)
0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	++ (3)	0 (3)	0 (2)
0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (3)	0 (2)	0 (2)
0 (3)	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*	*0 (2)*
0 (3)	0 (6)	0 (4)	0[illegible] (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (3)	0 (3)	0 (2)
0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	+++ (4)	0 (2)	0 (2)
[illegible] (2)	+++ (2)	0 (2)	0 (3)	0 (2)	0 (2)	+ (3)	0 (3)	++ (2)	0 (2)
(2)	++[illegible] (3)	++ (2)	++ (2)	0 (2)	0 (2)	++ (3)	+++ (2)	+++ (3)	++ (2)
(2)	+++ (2)	++ (2)	[illegible] (2)	+ (2)	+ (2)	+++ (2)	+++ (3)	++ (4)	++ (2)
0 (2)	++ (2)	0 (2)	0 (2)	0 (2)	+ (2)	+ (2)	+ (4)	+ (2)	++ (2)
[illegible] (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	0 (2)	+ (3)	0 (2)	0 (2)

pose a number of xenobiotic compounds sufficient to furnish a representative range of reactions were incubated with isolated strains of some common intestinal bacteria.

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1 Symbols. 0, none, + minor ++ moderate +++ extensive.

2 Number in brackets refers to number of experiments.

3 *See text.*

A aerogenes The extent of hydrolysis of 4-nitrocatechol sulphate seen in all incubates with *A aerogenes* was extremely small

While none of the bacteria hydrolyzed methyl gallate, *Bacillus* sp. nr 3 was found to give an unidentified metabolite in 2 of the 4 incubates. It gave a red to violet colour with fast blue B salt and had a Rf value slightly greater than resorcinol on thin-layer chromatograms developed with 20 / aq KCL – glacial acetic acid (100 1). The metabolite was not formed when gallic acid, pyrogallol or resorcinol were incubated and it was shown not to be 3,5-dihydroxybenzoic acid methyl ester

The reduction of 4-nitrobenzoic acid to 4-aminobenzoic acid which was seen with all bacteria except *Pseudomonas* proceeded via 4-hydroxyaminobenzoic acid. No evidence was obtained for the formation of 4-nitrosobenzoic acid. The 3 experiments with *Lactobacillus* sp nr 2 differed from all the others in that about half of the substrate was converted to the hydroxylamino derivative and only small amounts of 4-aminobenzoic acid were detected

Methyl red was reduced by all the bacteria tested. The extent of reduction was lowest with the strains of *Ps aeruginosa* which produced barely detectable amounts of the reduction products. *Bacillus* sp. nr 3 gave no reduction in one incubate and extensive reduction in the other incubate.

Discussion

It may be asked whether metabolism experiments with pure or mixed cultures of intestinal bacteria are an adequate reflection of the events occurring in the intestinal lumen. The *in vitro* experiments generally make use of a relatively simple incubation medium having a certain initial redox potential, whereas the conditions in the intestinal lumen are very complex both with regard to nutrients and the total microbial population. Also, the oxygen pressure in the center of the lumen has been found to be essentially zero while that at the intestinal wall is about a third of that found in air (SMITH 1969). The environmental conditions in the intestinal lumen are so poorly understood and difficult to reproduce that reactions which are known to occur there have not been reproduced *in vitro* Conversely reactions shown to take place *in vitro* may have little or no significance *in vivo* In spite of these reservations, several earlier reports from this laboratory (SCHELINE & LONGBERG 1965 SCHELINE 1966a & b INDAHL & SCHELINE 1971) and by others (SPATZ *et al.* 1967 GRIFFITHS 1970 SMITH & GRIFFITHS 1970 RENWICK & WILLIAMS 1969) have shown good correlation between *in vitro* and *in vivo* investigations.

All of the reactions examined in the present study have previously been carried out by incubates of mixed rat caecal microorganisms. However

in the case of acid yellow an azo dye which was found to be reduced by *Str faecalis* was a particular intestinal microorganism shown to carry out a particular reaction. While a few of the reactions in the present study were not carried out by any of the test microorganisms, the results show that most of the xenobiotics were metabolized by one or several of the microorganisms.

The findings that 4-methylumbelliferone glucuronide was hydrolyzed by *E. coli* and *Bacteroides* sp. correlate well with previous findings that high β-glucuronidase activities are found in the intestinal contents of many animal species (MARSH *et al.* 1952) Moreover *E coli* has been shown to possess β-glucuronidase activity (GLAZKO *et al.* 1952 BUEHLER *et al.* 1951). A similar property of *Bacteroides* is of considerable interest as this genus is a major group in the large intestine (DRASAR *et al* 1970) Recently HAWKSWORTH *et al.* (1971) found that the greatest amounts of β-glucuronidase are produced by *E. coli* while intermediate amounts are produced by Nagler-positive clostridia and *Bacteroides* However when the numbers of bacteria present in the rat intestine are taken into account it appears that glucuronides are hydrolyzed mainly by the non-sporing anaerobes and clostridia.

While numerous examples of glycoside hydrolysis by the intestinal flora are known (SCHELINE 1968d), few reports have appeared linking these with the metabolic activities of specific intestinal bacteria. A strain of *Str faecalis* and of *Lactobacillus salivarius salicinius* showing β-glucosidase activity have been reported by SPATZ *et al.* (1967) The present results also show that a strain of *Lactobacillus* is capable of hydrolyzing the β-glucoside linkage. However the other *Lactobacillus* studied was devoid of this effect. The finding that the reaction was carried out by *Pr vulgaris* and *A aerogenes* suggests that β-glucosidase activity may be of fairly widespread occurrence among the inhabitants of the intestinal flora. This belief is also supported by the recent report of HAWKSWORTH *et al.* (1971).

Hydrolysis of the amide bond was studied using 4-aminohippuric acid, 4-acetamidobenzoic acid, N^4-acetylsulphanilamide and succinylsulphathiazole. Only two of the microorganisms tested, *Str faecalis* and *A aerogenes* were found to possess amidase activity and this was restricted to either one or two of the test substances. It is of interest that, of the two N-acetyl compounds, only 4-acetamidobenzoic acid was hydrolyzed by *A aerogenes* Similar results were found earlier (SCHELINE 1968c) with mixed caecal cultures which sometimes hydrolyzed 4-acetamidobenzoic acid to 4-aminobenzoic acid while N^4-acetylsulphanilamide was not hydrolyzed. Hydrolysis of the amide bond in the glycine conjugate by *Str faecalis* was not unexpected in view of the earlier findings of NORMAN & GRUBB (1955) which showed that numerous enterococci hydrolyzed 4-aminohippuric acid and that this activity was also seen with bile acid conjugates. HILL & DRASAR (1967) have also shown that several strains of *Str faecalis* can hydrolyze taurocholate and other cholic

acid conjugates. The latter ability also appears to be fairly widespread among numerous strains of the strictly anaerobic genera of intestinal bacteria (HILL & DRASAR 1967 NAIR *et al* 1967)

Reduction of the double bond in caffeic acid by intestinal contents of several animal species was reported by BOOTH & WILLIAMS (1963) and this was later shown to be general reaction for cinnamic acid derivatives on incubation with rat caecal contents (SCHELINE 1968a) Also cinnamic acid was reduced to phenylpropionic acid when incubated with human faecal bacteria (HANSEN & CRAWFORD 1968) The present results indicate that several of the microorganisms tested can reduce the double bond in 3-hydroxycinnamic acid. The *Lactobacillus* sp. nr 2 was most active in this respect. WHITING & CARR (1959) reported that *Lactobacillus pastorianus* var *quinicus* reduced cinnamic acid, 4-hydroxy and 3 4-dihydroxycinnamic acids to the corresponding phenylpropionic acids.

Dehydroxylation by the intestinal microflora of several types of compounds including bile acids, heterocyclic nitrogen compounds and catechol derivatives is well known (SCHELINE 1968d). While several strains of bacteria have been implicated in the dehydroxylation reactions with the first group of compounds (GUSTAFSSON *et al* 1966 & 1968 MIDTVEDT 1967 MIDTVEDT & NORMAN 1968) little is known of the bacteria responsible for removal of hydroxyl groups in the other groups of compounds and the present results do not give any positive information on this subject. Thus, the finding of PERETZ SILVA *et al* (1966) who isolated a strain of *Pseudomonas* from rat faeces capable of dehydroxylating caffeic acid, is the only report available which implicates a specific microorganism with this reaction. PEPPERCORN & GOLDMAN (1971) have shown, however that dihydrocaffeic acid is dehydroxylated to *m*-hydroxyphenylpropionic acid by a mixed culture of *E coli* and *Str faecalis* var. *liquifaciens* No reaction was observed when the substrate was incubated with either organism alone. Surprisingly no dehydroxylation occurred when a cell suspension of both cell types taken directly from pure cultures was used.

Earlier work from this laboratory has shown that the decarboxylation of 4-hydroxycinnamic acids observed when these compounds are incubated with mixed cultures of rat caecal microorganisms is also brought about by two strains of *Bacillus* isolated from the rat caecum (INDAHL & SCHELINE 1968) The strain of *Bacillus* isolated in the present study appears to have similar properties. *A aerogenes* however has a more extensive range of activity in that both *p*-hydroxylated benzoic and cinnamic acid derivatives were decarboxylated. FINKLE *et al.* (1962) reported that several strains of *A aerogenes* decarboxylated *p*-hydroxycinnamic acid derivatives. Decarboxylation of caffeic acid has recently been reported to be carried out by *Streptococcus faecium* (PEPPERCORN & GOLDMAN 1971). Thus, the intestinal decarboxyl-

of *p*-hydroxylated benzoic and cinnamic acid derivatives is explainable in terms of their metabolism by particular intestinal bacteria. However the *decarboxylation* of the corresponding phenylacetic acid derivatives observed with mixed caecal cultures (SCHELINE 1968a) has not been demonstrated with pure cultures.

Reduction of the nitro compound and the two azo compounds was carried out by the majority of the microorganisms tested although acid yellow a highly polar azo compound, was extensively reduced only by *Str faecalis* Some of the characteristics of the enzyme system from *Str faecalis* have been reported (SCHELINE *et al* 1970) The finding that azo reduction is fairly general among intestinal bacteria is in accordance with several earlier reports (see WALKER 1970).

The present results furnish, as mentioned above, several positive correlations between a particular intestinal microorganism and the metabolism of a specific xenobiotic compound. However it should be kept in mind that the predominant intestinal bacteria belong to the non-sporing strictly anaerobic groups which are not fully represented in this study Because of their greater numbers, their actual significance with regard to the metabolism of xenobiotics may be far greater than that of the many less abundant members of the intestinal microflora which often show greater specific enzyme activities. An example of this has recently been given by HAWKSWORTH *et al.* (1971) Another point of interest is the fact that the intestinal metabolism of a compound may consist of a series of reactions and that these may be carried out by several microorganisms. Also a single metabolic step may require the interplay of more than one microorganism as shown by PEPPERCORN & GOLDMAN (1971) in the dehydroxylation of caffeic acid.

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From the Department of Microbiology Dental Faculty
University of Oslo Oslo, Norway

The Herbicide 2, 4-Dichlorophenoxyacetic Acid
II Triglyceride Accumulation in L Cells

By

Jan Kolberg, Kristen Helgeland and Jon Jonsen
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Abstract: L 929 cells in monolayer cultures exposed to 500 μg/ml of 2,4-dichlorophenoxyacetic acid have been found to accumulate triglycerides. Labelling experiments were performed with ^{3}H-palmitate and ^{14}C-acetate. The incorporation of ^{3}H and ^{14}C into total cell lipids of treated cells was about 3 times that in the control cells. The increase of ^{3}H was mainly recovered in the triglyceride fractions both from whole cells and in isolated cytoplasmic particles. The specific activity of the triglycerides with regard to tritium remained similar whereas the specific activity with regard to ^{14}C was only one-fourth in the treated cells as compared to the control cells. This suggested that the fatty acids of the accumulated triglycerides were largely derived from the growth medium.

Key words: Herbicide – L cells – accumulation of triglycerides.

Previous studies have shown that 2,4-dichlorophenoxyacetic acid (2,4-D) inhibits the growth of L cells in monolayer cultures and induces a transient accumulation of cytoplasmic particles which stain with lipid soluble dyes (Kolberg *et al.* 1971).

It has been found that 80–95 / of the fatty acids incorporated into cellular glycerides and phospholipids of tissue culture cells were derived from fatty acids of the serum added to the medium (reviewed by Rothblat (1969)). To test if the lipid accumulation induced by 2,4-D was due to an increased uptake of fatty acids, the incorporation of ^{3}H-palmitate was measured. An effect of 2,4-D on the endogenous synthesis of fatty acids was evaluated by adding ^{14}C-acetate to the medium.

Materials and Methods

A reference preparation of 2,4-D (purity > 99 X gas chromatography by the manufacturer) was gift from Køge Chemical Works Ltd., Køge, Denmark, and the Na-K

salt was prepared as described previously (KOLBERG *et al.* 1971) Tripalmitin (approximately 99 %) and cholesterol (99+ %) were purchased from Sigma Chemicals. Sodium 1-^{14}C-acetate (61 mci/mmol), 9,10-^{3}H-palmitic acid (500 mci/mmol), 1,2-^{3}H-n-hexadecane and 1-^{14}C-n-hexadecane were supplied by The Radiochemical Centre, Amersham, U K. Kiesel gel H nach Stahl was delivered by E. Merck, A.G. Freshly distilled chloroform, methanol, diethylether and petrol ether were used throughout the experiments.

Cell culture techniques

Mouse fibroblasts, strain L 929 were cultured in Eagle s Minimum Essential Medium supplemented with 10 % calf serum, penicillin and streptomycin as described previously (KOLBERG *et al* 1971). Cell countings were performed in Bürker haemocytometer Petri dishes, 100 × 20 mm (Falcon Plastic Inc.) were seeded with 2.0 × 10^6 cells suspended in 25 ml medium. After treatment with 2,4-D, the cell layers were washed twice with 5 ml ice-cold medium without serum and harvested by scraping with a rubber policeman.

Isolation of lipids

The lipid particles were isolated according to the method of MACKENZIE *et al.* (1966). The cell layers were washed twice with ice-cold 0.15 M NaCl and once with ice-cold distilled water The cells were harvested by scraping with a rubber policeman into ice-cold distilled water and the cell suspension was forced 5 times through a 25 gauge hypodermic needle. The resulting homogenate was centrifuged in a cellulose nitrate tube at 35,000 r.p.m. for 30 min. at 4 in a SW-41 rotor in a Spinco Ultracentrifuge. The top layer containing the lipid particles, was removed and re-centrifuged. The particles were then lyophilized and re-dissolved in chloroform-methanol (2:1, v/v). The pelleted cell residue after the first centrifugation was extracted by means of a five-step treatment with chloroform-methanol (1 1, v/v) according to WEINSTEIN *et al.* (1969). The same procedure was used for extraction of whole cells.

Separation of lipids

The lipid extracts were washed once with 0.2 volume of water at 4 (FOLCH *et al.* 1957), evaporated to dryness under a stream of nitrogen, and re-dissolved in chloroform-methanol (2:1, v/v).

Thin-layer chromatography (TLC) was performed on silica gel H applied as a 0.5 mm layer on glass plates. Before use, the plates were developed in chloroform-methanol (4 1, v/v) to remove impurities, and activated for 30 min. at 110° Aliquots of the lipid extracts were applied in bands under a stream of nitrogen. Development was performed with petrol ether (b.p. 60–70°)-diethylether-acetic acid (90:10:1 v/v) in nitrogen atmosphere. The plates were developed twice with intermediate drying and the lipids visualized by exposure to iodine vapour

The outlined segments of silica gel were sucked into Pasteur pipettes plugged with glass wool. Cholesterol and triglycerides were eluted with chloroform, phospholipids with methanol-HCl (19:1, v/v)

Chemical analysis.

Triglycerides were determined by the method of VAN HANDEL & ZILVERSMIT (1957), cholesterol by the procedure of STADTMAN (1957) Phosphorus was measured by the method of BARTLETT (1959), according to MARINETTI (1962) The phospholipid content was calculated by multiplying the phosphorus value by a factor of 25

Radioactive labelling

Labelling was performed with 0.1 µci/ml medium of ^{3}H-palmitate and 0.025 µci/ml of ^{14}C-acetate for a period of 24 hrs. The purchased ^{3}H-palmitic acid was extracted from the benzene-hexane solution into alkaline 50 % ethanol, added to serum and incubated for 30 min. at 37 The serum was then used to make the complet medium, to which sodium ^{14}C-acetate was added. The ethanol concentration in the final medium was 0.04 %

Liquid scintillation counting

Lipid-containing TLC (0.25 mm layer) spots were scraped into polyethylene vials. To solubilize polar lipids adsorbed to the silica particles (SNYDER 1964), 1 ml water was added to 15 ml dioxane based scintillation liquid (400 g naphtalene, 25 g PPO 0.5 g POPOP 2 l dioxane, 2 l xylene, and 1 l ethanol) The cell residue was dissolved in 1 N-NaOH, 50 µl of this solution heated with 300 µl Hyamine 10−X for 10 min. at 70 and counted in the same solution, but without addition of water

Counting was performed in Packard Tri-Carb spectrometer model 3365 operated at 3 and with ^{3}H-and ^{14}C-n-hexadecane as standards.

Results

By phase-contrast microscopy of cells treated with 500 µg 2,4-D/ml medium, the number of lipid particles was found to be at a maximum within 24 hrs. In a representative experiment, the cell lipids increased from 57 to 127 µg/10^{6} cells during this period (table 1). Triglycerides accounted for 61 µg, cholesterol for 1.3 µg and phospholipids for 7 µg of this increase.

The incorporation of ^{3}H from palmitate into the lipid fraction increased about 3 times after treatment with 2,4-D The amount of ^{3}H in the non-lipid fraction, however was unchanged and less than 1 / of the total incorporation. On the other hand, the incorporation of ^{14}C from acetate increased about 3 and 5 times in the lipid and non-lipid fractions, respectively About 85 / of the ^{14}C activity was recovered from the lipid fractions in both treated and control cells.

The increased incorporation of ^{3}H into the lipids was recovered from the triglyceride fraction, whereas the increase in incorporation of ^{14}C was found both in the triglyceride and the cholesterol fractions. The specific activity of triglycerides with regard to ^{3}H remained unchanged, whereas the specific activity with regard to ^{14}C was only 27 / in the treated cells compared to the control cells. The specific activity of both ^{3}H and ^{14}C in phospholipids decreased to about 50 / of that in the control cells.

Lipid particles were isolated from disrupted cells. Seventy-three / of the increased incorporation of ^{3}H into total cell triglycerides was recovered in this fraction, whereas 4 / was found in the pellet (table 2) The recovery of the ^{3}H-label after disruption of the cells was 100 / for the control and 75 / for the treated cells. The lower recovery for the latter is probably d

Table 1

Incorporation of ^{3}H-palmitate and ^{14}C-acetate into different cell fractions by exposure to 500 µg 2,4-D/ml for 24 hrs.

	^{3}H		^{14}C	
	Control	2,4-D	Control	2,4-D
Total lipids				
d.p.m.	590,000	1,100,000	6,600	22,000
µg**	57	127	57	127
Triglycerides				
d.p.m.	69 700	766,000	924	3,540
µg	4.6	66	4.6	66
Spec. act.	15,000	12,000	200	54
Cholesterol				
d.p.m.	3,970	3,300	1,890	10,000
µg	4.7	6.0	4.7	6.0
Spec. act.	840	550	400	1,700
Phospholipids				
d.p.m.	286,000	191,000	3,070	1,380
µg	48	55	48	55
Spec. act.	6,000	3,500	64	25
Recovery***	92	87	89	68
Cell residue after lipid extraction				
d.p.m.	3,800	3,400	1,100	5,300

Measured in duplicate cultures. The results are given per 10^6 cells.
The growth inhibition due to 2,4-D was found to be 60 % by cell counts.
** Sum of analyzed lipid fractions.
*** Percentage radioactivity from the lipid extracts recovered in the analyzed lipid fractions.

to some loss of lipid particles due to manipulations during the isolation procedure

Discussion

Previously we have found that L 929 cells exposed to 2,4-D accumulate lipid-containing particles in the cytoplasm (KOLBERG *et al.* 1971). In the present experiments chemical analysis showed that cells treated with 500 µg 2,4-D/ml for 24 hrs contained about twice the amounts of lipids in the control cells, mainly due to an increase in the triglyceride fraction.

The incorporation of ^{3}H from ^{3}H-palmitate into total cell lipids increased

Table 2

Incorporation of ^3H-palmitate into lipids from whole cells and cell fractions by exposure to 500 µg 2,4-D/ml for 4 hrs.

	Whole cells		Lipid particle fraction		Pellet fraction	
	Control	2,4-D	Control	2,4-D	Control	2,4-D
Total lipids	200,000	600,000	19,000	280,000	180,000	170,000
Triglycerides	34,000	380,000	16,000	270,000	6,900	21,000

Measured in duplicate cultures. The results are given as dpm/10^6 cells.

about three times after treatment with 2,4-D This increase was mainly recovered from the triglyceride fraction. The specific activity of the tritium label in the triglyceride fractions from cells exposed to 2,4-D and control cells was, however similar As MACKENZIE *et al.* (1967) and HOWARD & KRITCHEVSKY (1969) have reported that fatty acids in cell lipids are not biosynthesized but derived from the medium, this similarity in specific activity indicates that the fatty acids of the accumulated triglycerides are, to a large extent, obtained from the medium.

When lipid particles were isolated from disrupted cells, about three-fourths of the increased incorporation of tritium into triglycerides were recovered from these particles. This indicates that the main part of the ac cumulated triglycerides are confined to the observed cytoplasmic particles (KOLBERG *et al.* 1971)

During the exposure to 2,4-D the incorporation of ^{14}C from ^{14}C-acetate into triglycerides also increased. This could be due to the increased require ment for glycerol in the triglyceride production. As the specific activity of ^{14}C in triglycerides from the treated cells was only about one-fourth of that in the control cells, an increased endogenous synthesis of fatty acids seems to play a minor role in the lipid accumulation. The increased incorporation of ^{14}C into both cholesterol and cell residue may be explained by the increased uptake of acetate and/or by a higher synthesizing activity in cells exposed to 2,4-D

In both the control and treated cells the amount of ^3H in the cell residues after lipid extraction was less than 1 / of that in the lipid fraction. This suggests that the fatty acids are not readily metabolized during the incubation period of 24 hrs. This is consistent with the studies of GEYER (1967) using L cells. About 15 / of the incorporated ^{14}C-acetate into both treated and non-treated cells was found in the cell residues. This shows that this precursor entered the general cell metabolism to a higher degree than palmitate.

Equal amounts of phospholipids were found in the treated and control cells, whereas the specific activities of the precursors in this fraction in the presence of 2,4-D was about 50 / of that in the control cultures. This reduced incorporation is in accordance with the growth inhibition, about 60 / being observed in the presence of 2,4-D

The accumulation of lipid particles in cultured mammalian cells may be due to an increased incorporation of exogenous fatty acids (GEYER 1967 MACKENZIE *et al* 1967 MOSKOWITZ 1967) The higher lipogenic activity of rabbit compared to horse serum has been shown to be related to a higher concentration of albumin per µmol fatty acids in horse serum (MACKENZIE *et al*, 1970) SPECTOR *et al* (1965) showed that the uptake of palmitate by Ehrlich ascites cells depends on the molar ratio of palmitate to albumin in the medium. Chlorophenoxyisobutyrate (CPIB), which is structurally related to 2,4-D and used in the treatment of hyperlipidemia, has been found to increase the uptake of fatty acids into Ehrlich ascites cells (SPECTOR & SOBOROFF 1971). This was suggested to be due to a displacement of fatty acids from strong to weaker binding sites on human plasma albumin, thereby making the fatty acids more available to the cells. Whether part of the effect of 2,4-D may be due to a similar mechanism is not known. The gel filtration experiment by ERNE (1966) indicates a weak binding of 2,4-D to plasma proteins

The increased incorporation of both ^{3}H-palmitate and ^{14}C-acetate found in our experiments suggests, however that 2,4-D might have an effect on the permeability of the plasma membrane.

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From the Department of Pharmacology and Toxicology H. Lundbeck & Co. A/S, 7–9 Ottiliavej, DK 2500 Copenhagen-Valby Denmark

Antagonism of Methylphenidate-Induced Stereotyped Gnawing in Mice

By

V Pedersen and A. V Christensen

(Received April 14 1972, Accepted June 22, 1972)

Abstract It was observed by chance that methylphenidate induces an intense stereotyped gnawing in mice. Amphetamine too, induces stereotyped behaviour in mice, but this behaviour is mainly characterized by licking and rapid movements of the head and forelegs. Thus, the behavioural effects of amphetamine and methylphenidate in mice seem to be different under certain experimental conditions. The effect of methylphenidate was not blocked by pretreatment with dopamine-β-hydroxylase inhibitors and phenoxybenzamine, while the dopamine receptor blocking neuroleptics caused a complete inhibition. These findings support the view that brain dopamine is associated with production of stereotyped behaviour Pretreatment with the tyrosine hydroxylase inhibitor d,l-α-methyltyrosine methylester (H 44/68) 1, 2 or 3 hours before methylphenidate caused no or only partial inhibition of the stimulant effect. Pretreatment with H 44/68 5 hours before methylphenidate completely inhibited the development of compulsive gnawing, indicating that the antagonistic effect of H 44/68 seems to be correlated with gross depletion of catecholamines from the brain. Reserpine too, was found to inhibit the effect of methylphenidate. A series of centrally acting drugs were tested for antagonistic effect of methylphenidate and only the neuroleptics proved to be active. A classification of 20 neuroleptics with respect to their methylphenidate antagonistic effect is presented The clinically potent neuroleptics were active in very low doses, but the weaker "basal" neuroleptics like chlorpromazine and thioridazine also showed clearcut effect.

Key-words: Psychopharmacology – methylphenidate catecholamines – compulsive behaviour – tranquilizing agents.

The stereotyped behaviour induced by amphetamine in rats is usually considered to be an experimental model of psychotic behaviour and inhibition of this behaviour is regarded as being a very selective test for screening of neuroleptic activity (JANSSEN *et al* 1965 & 1967) Furthermore it has been shown that the effect of amphetamine in rats is inhibited by α-methyltyrosine but not by reserpine (WEISSMAN *et al.* 1966 RANDRUP & MUNKVAD 1966

SCHELL-KRÜGER 1971), indicating that the effect of amphetamine is dependent on a continuous synthesis of catecholamines in the brain.

By chance it was observed in our laboratories that methylphenidate in mice induces an intense gnaw-compulsion syndrome (PEDERSEN & CHRISTENSEN 1971) The same observation has, however been made earlier by THIR & SCHRAMM (1962). Furthermore dexamphetamine has also been described as inducing stereotypies in mice in doses of 7.5–15 mg/kg subcutaneously (RANDRUP & MUNKVAD 1967 MENGE & BRAND 1971) while lower doses only cause increased locomotor activity (SVENSSON 1970).

The purpose of the present study has been to investigate whether the effect of methylphenidate in mice is dependent on an interaction with brain catecholamines. Moreover we found it of interest to examine whether the inhibition of methylphenidate-induced stereotyped gnawing in mice may be a useful test for the screening of neuroleptic activity

Materials and methods

Male mice (NMRI/BOM SPF) in the weight range of 18–25 g were used throughout. I order to determine a challenging dose of methylphenidate hydrochloride, increasing doses of the drug were given subcutaneously Immediately after the injection the mice were placed in observation cages, two mice in each cage, and the excitatory effects were observed during a one hour period. The cages consisted of 30 cm high perspex boxes (12×25 cm) without bottom or lid. During the experiments the cages were placed on corrugated paper the corrugations of which faced upwards.

In the subsequent experiments, in which the effect of different drugs on methylphenidate-induced stereotyped gnawing was studied, the following procedure was used. After pretreatment with the drug under investigation (time schedule and number of animals per dose given under results) the mice were given a subcutaneous injection f methylphenidate hydrochloride, 60 mg/kg, and were placed in the cages described above, two mice in each cage, for exactly one hour For each dose level the number of pairs that did not bite the corrugated paper was counted. ED50 values were determined by *probit analyses*, using log dose scale, and the calculations were *done by* means of an IBM 1130 computer system.

The following compounds were given as aqueous solutions: Methylphenidate hydrochloride (ritalin®), amphetamine sulphate, H 44/68 (d,l-α-methyltyrosine methylester hydrochloride, Hassle), DDC (sodium diethyldithiocarbamate), *p*-chlorophenylalanine methylester hydrochloride, amitriptyline hydrochloride, imipramine hydrochloride, atropine sulphate, scopolamine hydrobromide, cyproheptadine hydrochloride, diphenhydramine hydrochloride, metoclopramide hydrochloride, acepromazine maleate, clopenthixol dihydrochloride, chlorpromazine hydrochloride, chlorprothixen hydrochloride, flupenthixol dihydrochloride, fluphenazine dihydrochloride, levopromazine hydrochloride, methylperone hydrochloride, tetrabenazine hydrochloride, thioridazine hydrochloride and trifluoperazine dihydrochloride.

Solutions of diazepam (valium®) prochlorperazine (stemetil®) and reserpine (serpasil®) were prepared from the commercially vailable solutions. The *following substances* were dissolved by adding the equivalent amount of 0.1 N hydrochloric acid. ch diazepoxid (clopoxidum NFN), phenoxybenzamine (benzylytum NFN), pe

Table 3

Protection against methylphenidate-induced compulsive gnawing in mice.

Compound	Number of pairs per dose	ED50 mg/kg i.p.	95 % confidence interval
Fluphenazine	5	0.04	0.02 – 0.09
Pimozide	3	0.05	0.03 – 0.09
Perphenazine	3	0.06	0.04 – 0.09
Haloperidol	3	0.06	0.04 – 0.09
Loxapine	6	0.10	0.05 – 0.22
Trifluoperazine	6	0.12	0.08 – 0.18
Flupenthixol	12	0.13	0.08 – 0.22
Clothiapine	3	0.14	0.09 – 0.22
Thiothixene	3	0.44	0.30 – 0.64
Chlorprothixene	15	0.71	0.27 – 1.84
Levopromazine	3	1.4	0.60 – 3.3
Clopenthixol	5	1.7	0.96 – 3.0
Methylperone	3	2.2	1.3 – 3.7
Prochlorperazine	3	2.5	1.3 – 5.2
Chlorpromazine	5	4.0	2.5 – 6.4
Thioridazine	10	5.4	3.7 – 7.9
Acepromazine	3	11.1	6.7 – 18.3
Reserpine	3	2.5	1.2 – 5.2
Tetrabenazine	3	40.5	21.4 – 76.3
Clozapine	3	> 160	–

All neuroleptics were administered intraperitoneally 2 hours before the injection of methylphenidate.

The experiments with reserpine show that high doses (10 mg/kg, 5 hrs) of reserpine completely antagonized the stimulant effects of methylphenidate. Of the mice pretreated with 5 mg/kg of reserpine only one pair showed weak signs of stereotyped gnawing.

Effect of neuroleptics

The neuroleptic drugs were administered intraperitoneally 2 hours before methylphenidate. The ED50 values obtained are presented in table 3. From the table it appears that all the neuroleptics tested except clozapine showed a methylphenidate antagonistic effect and that in this test the clinically potent neuroleptics also proved to be the most potent ones. The ED50 of chlorprothixene is surprisingly low and it was observed that the dose-effect curve for this compound was rather flat as compared with those obtained with the other neuroleptics. This is reflected in the confidence interval which is rather wide in spite of the large number of animals.

SCHEEL-KRÜGER 1971) indicating that the effect of amphetamine is dependent on a continuous synthesis of catecholamines in the brain.

By chance it was observed in our laboratories that methylphenidate in mice induces an intense gnaw-compulsion syndrome (PEDERSEN & CHRISTENSEN 1971). The same observation has, however, been made earlier by THER & SCHRAMM (1962). Furthermore dexamphetamine has also been described as inducing stereotypies in mice in doses of 7.5–15 mg/kg subcutaneously (RANDRUP & MUNKVAD 1967, MENGE & BRAND 1971) while lower doses only cause increased locomotor activity (SVENSSON 1970).

The purpose of the present study has been to investigate whether the effect of methylphenidate in mice is dependent on an interaction with brain catecholamines. Moreover we found it of interest to examine whether the inhibition of methylphenidate-induced stereotyped gnawing in mice may be a useful test for the screening of neuroleptic activity.

Materials and methods

Male mice (NMRI/BOM SPF) in the weight range of 18–25 g were used throughout.

In order to determine a challenging dose of methylphenidate hydrochloride, increasing doses of the drug were given subcutaneously. Immediately after the injection the mice were placed in observation cages, two mice in each cage, and the excitatory effects were observed during a one hour period. The cages consisted of 30 cm high perspex boxes (12×25 cm) without bottom or lid. During the experiments the cages were placed on corrugated paper, the corrugations of which faced upwards.

In the subsequent experiments, in which the effect of different drugs on methylphenidate-induced stereotyped gnawing was studied, the following procedure was used. After pretreatment with the drug under investigation (time schedule and number of animals per dose given under results) the mice were given a subcutaneous injection of methylphenidate hydrochloride, 60 mg/kg, and were placed in the cages described above, two mice in each cage, for exactly one hour. For each dose level the number of pairs that did not bite the corrugated paper was counted. ED50 values were determined by probit analysis, using a log dose scale, and the calculations were done by means of an IBM 1130 computer system.

The following compounds were given as aqueous solutions. Methylphenidate hydrochloride (ritalin®), amphetamine sulphate, H 44/68 (d,l-α-methyltyrosine methylester hydrochloride, Hässle), DDC (sodium diethyldithiocarbamate), p-chlorophenylalanine methylester hydrochloride, amitriptyline hydrochloride, imipramine hydrochloride, atropine sulphate, scopolamine hydrobromide, cyproheptadine hydrochloride, diphenhydramine hydrochloride, metoclopramide hydrochloride, acepromazine maleate, clopenthixol dihydrochloride, chlorpromazine hydrochloride, chlorprothixene hydrochloride, flupenthixol dihydrochloride, fluphenazine dihydrochloride, levopromazine hydrochloride, methylperone hydrochloride, tetrabenazine hydrochloride, thioridazine hydrochloride and trifluoperazine dihydrochloride.

Solutions of diazepam (valium®), prochlorperazine (stemetil®) and reserpine (serpasil®) were prepared from the commercially available solutions. The following substances were dissolved by adding the equivalent amount of 0.1 N hydrochloric acid: chlordiazepoxid (clopoxidum NFN), phenoxybenzamine (benzylytum NFN), perphenazine

and thiothixene. Pimozide and haloperidol were dissolved in 0.1 N tartaric acid and the solutions were diluted with water

FLA 63 (bis(4-methyl 1-homopiperazinyl-thiocarbonyl) disulfide, Hässle) was dissolved in 1 N hydrochloric acid and after dilution with water the pH was adjusted to 5 by means of sodium hydroxide.

Clothiapine, clozapine, loxapine and l-DOPA were administered as suspensions in 0.5 % methylcellulose.

Methylphenidate and amphetamine were administered subcutaneously and all other compounds were given intraperitoneally Injection volume 10 ml/kg. The doses given refer to the form indicated above.

Results

Behavioural effects of different doses of methylphenidate.

Methylphenidate was given in doses ranging from 40 to 65 mg/kg subcutaneously and all doses caused excitatory responses A few minutes after the injection the animals showed increased locomotor activity characterized by continuous running and jumping. At the higher dose levels this behaviour changed into a typical gnaw-compulsion within 10–15 minutes and all the animals began to bite the corrugated paper After the lower doses licking movements were seen, but only some of the mice showed stereotyped gnawing (see table 1) During the period with stereotyped gnawing, forward locomotion and rearing were occasionally seen while normal grooming activity was completely absent. In a single experiment (60 mg/kg) the observation period was extended to two hours and it was observed that normal behaviour gradually returned after 1–2 hours.

The effect of 20 and 40 mg/kg subcutaneously of amphetamine sulphate was investigated in a similar experiment using 5 pairs of mice at each dose level. Both doses caused a markedly increased locomotor activity and within 40–50 minutes the mice intermittantly showed stereotyped behaviour most pronounced in the 40 mg/kg group Very often the mice stood on their

Table 1

Methylphenidate-induced compulsive gnawing in mice.

Dose mg/kg s. c.	Number of pairs dosed	Number of pairs biting
40	5	2
45	5	4
50	5	4
55	5	5
60	5	5
65	5	5

hindlegs licking and occationally making attempts to bite the walls of the cages or sat in a characteristic posture with hunched back showing rapid stereotyped movements of the head and forelegs. Grooming activity was frequently observed, probably because of profuse salivation. In sharp contrast to the methylphenidate treated animals the mice treated with amphetamine did not bite the corrugated paper within the 2 hours observation period. Furthermore it should be noted that stereotyped movements of the head and forelegs were never observed in the animals treated with methylphenidate

For the subsequent experiments 60 mg/kg of methylphenidate was chosen as the challenging dose for provoking the gnaw-compulsion syndrome.

Effect of different drugs interfering with brain amines

The results have been summarized in table 2. Pretreatment with the tyrosine hydroxylase inhibitor H 44/68 300 mg/kg, 5 hours before the administration of methylphenidate completely inhibited the development of the compulsive gnawing while the 2 and 3 hours periods of pretreatment caused only a partial inhibitory effect. Pretreatment with H 44/68 1 hour before challenge had no inhibitory effect. Furthermore a dose of 200 mg/kg of 1-DOPA completely reactivated the animals pretreated with H 44/68 5 hours before methylphenidate administration.

The mice pretreated with DDC were heavily sedated but in spite of this, methylphenidate provoked an intense gnaw-compulsion syndrome. FLA 63 too, failed to inhibit the effect of methylphenidate. The 5-HT synthesis inhibitor *p*-chlorophenylalanine did not affect this behaviour

Table 2

Protection against methylphenidate-induced compulsive gnawing in mice.

Pretreatment	Number of pairs dosed	Number of pairs biting
H 44/68 300 mg/kg i.p., 1 hr	5	5
H 44/68 300 mg/kg i.p. 2 hrs	5	3
H 44/68 300 mg/kg i.p., 3 hrs	5	1
H 44/68 300 mg/kg i.p., 5 hrs	5	0
H 44/68 300 mg/kg i.p. 5 hrs + 1 DOPA 200 mg/kg i.p., 1 hr	5	5
DDC, 500 mg/kg i.p. 6, 4 and 1 hrs	5	5
FLA 63 50 mg/kg i.p., 4 hrs	5	5
p-chlorophenylalanine methylester 100 mg/kg i.p., 72, 48 and 18 hrs	5	5
Reserpine 5 mg/kg i.p., 5 hrs	5	1
Reserpine 10 mg/kg i.p., 5 hrs	5	0

The animals showed only very weak signs of compulsive gnawing.

Table 3

Protection against methylphenidate-induced compulsive gnawing in mice.

Compound	Number of pairs per dose	ED50 mg/kg i.p.	95 % confidence interval
Fluphenazine	5	0.04	0.02 – 0.09
Pimozide	3	0.05	0.03 – 0.09
Perphenazine	3	0.06	0.04 – 0.09
Haloperidol	3	0.06	0.04 – 0.09
Loxapine	6	0.10	0.05 – 0.22
Trifluoperazine	6	0.12	0.08 – 0.18
Flupenthixol	12	0.13	0.08 – 0.22
Clothiapine	3	0.14	0.09 – 0.22
Thiothixene	3	0.44	0.30 – 0.64
Chlorprothixene	15	0.71	0.27 – 1.84
Levomepromazine	3	1.4	0.60 – 3.3
Clopenthixol	5	1.7	0.96 – 3.0
Methylperone	3	2.2	1.3 – 3.7
Prochlorperazine	3	2.5	1.3 – 5.2
Chlorpromazine	5	4.0	2.5 – 6.4
Thioridazine	10	5.4	3.7 – 7.9
Acepromazine	3	11.1	6.7 18.5
Reserpine	3	2.5	1.2 5.2
Tetrabenazine	3	40.5	21.4 76.5
Clozapine	5	> *160*	

All neuroleptics were administered intraperitoneally 2 hours before the injection of methylphenidate.

The experiments with reserpine show that high doses (10 mg/kg, 5 hrs) of reserpine completely antagonized the stimulant effects of methylphenidate. Of the mice pretreated with 5 mg/kg of reserpine only one pair showed weak signs of stereotyped gnawing.

Effect of neuroleptics

The neuroleptic drugs were administered intraperitoneally 2 hours before methylphenidate. The ED50 values obtained are presented in table 3. From the table it appears that all the neuroleptics tested except clozapine showed a methylphenidate antagonistic effect and that in this test the clinically potent neuroleptics also proved to be the most potent ones. The ED50 of chlorprothixene is surprisingly low and it was observed that the dose-effect curve for this compound was rather flat as compared with those obtained with the other neuroleptics. This is reflected in the confidence interval which is rather wide in spite of the large number of animals.

Table 4

Protection against methylphenidate-induced compulsive gnawing in mice.

Compound	Highest dose mg/kg i.p.	Number of pairs tested	Number of pairs protected
Phenoxybenzamine	20	3	0
Chlordiazepoxyd	20	5	0
Diazepam	20	5	0
Amitriptyline	40	5	0
Imipramine	40	5	0
Atropine	2.5	5	0
Scopolamine	2.5	5	0
Diphenhydramine	10	5	0
Cyproheptadine	10	3	0
Metoclopramide	80	3	1

All compounds were administered intraperitoneally 2 hours before the injection of methylphenidate.

Effect of different centrally acting drugs

All the compounds were administered intraperitoneally 2 hours before methylphenidate, and the results are presented in table 4. It can be seen that none of the compounds investigated in this series yielded protection against methylphenidate-induced compulsive gnawing. With scopolamine a certain potentiation rather than a protection was observed. Metoclopramide which has been described as a potent anti-emetic in humans (JONES 1968, GYLDING-SABROE & HATTEL 1969) failed to inhibit the effect of methylphenidate. Metoclopramide possesses weak anti-amphetamine and anti-apomorphine effects in rats (JANSSEN *et al.* 1967) and protects against vomiting but not against stereotypy induced by apomorphine in dogs (NYMARK 1972).

Discussion

Methylphenidate and amphetamine have been shown to induce very similar behaviourally excitatory effects in rats (SCHEEL-KRÜGER 1971), however minor differences have been reported by FOG (1969). Under the experimental conditions described in this paper, the behavioural effects of the two compounds in mice were different, although both compounds caused stereotypies. The amphetamine-induced stereotyped behaviour is mainly characterized by licking and rapid movements of the head and forelegs. The stereotypies induced by methylphenidate consist almost exclusively of compulsive gnawing. Our observations disagree with those of MENGE & BRAND (1971) who reported that amphetamine and methylphenidate produce similar be-

havioural symptoms in mice. The discrepancy may be due to differences in the experimental conditions.

The effect of methylphenidate in mice was not blocked by inhibition of dopamine-β-hydroxylase by DDC and FLA 63 or by pretreatment with phenoxybenzamine which has been described to possess a higher capacity for blocking central rather than peripheral NA receptors (BOLME & FUXE 1971 ANDÉN et al 1967) In contrast, the DA receptor blocking neuroleptics completely inhibited the development of stereotyped gnawing after the administration of methylphenidate. These findings support the view that brain dopamine is associated with the production of stereotyped behaviour (ERNST 1967 SCHEEL KRÜGER & RANDRUP 1967)

The hyperactivity induced by amphetamine in mice is not inhibited by reserpine, but is easily antagonized by low doses of α-methyltyrosine indicating that the effect of amphetamine is not correlated with total brain levels of catecholamines (CA) but is dependent on the presence of newly synthesized CA (WEISSMAN et al. 1966 SVENSSON 1970). In contrast, the present results show that the effect of methylphenidate in mice is completely inhibited by reserpine. Pretreatment with H 44/68 (300 mg/kg) 1 2 or 3 hours before methylphenidate caused no or only partial inhibition of the stimulant effect while pretreatment for 5 hours, caused a more marked decrease in brain CA, which completely inhibited the development of hyperactivity and compulsive gnawing. Thus, the antagonism of the behavioural effects of methylphenidate by H 44/68 seems to be correlated with gross depletion of CA from the brain.

According to the hypothesis that catecholamines are found in at least two different neuronal pools, a small, functional (reserpine-resistant) pool and a larger nonfunctional (reserpine-sensitive) storage pool (CARLSSON 1966 RECH et al 1968 and others) the present study indicates that the methylphenidate-induced stereotyped gnawing in mice depends on an interaction with a reserpine-sensitive pool of dopamine. The same conclusion has been drawn by SCHEEL KRÜGER (1971) who in rat experiments found that reserpine completely inhibited all the behavioural and biochemical effects of methylphenidate.

As expected the neuroleptic drugs inhibited the development of methylphenidate-induced compulsive gnawing and of all the types of drugs investigated, only the neuroleptics proved to be active in this respect (tables 3 and 4) A classification of a series of 20 neuroleptics with regard to their methylphenidate antagonistic effect is presented in table 3 It appears that the clinically potent neuroleptics are effective in the methylphenidate test at very low dose levels. Also the weaker "basal" neuroleptics like chlorpromazine and thioridazine show a clearcut effect. The only neuroleptic drug without any methylphenidate antagonistic effect is clozapine which by

us and others (STILLE *et al.* 1971) has also been found to be devoid of any anti-amphetamine and anti-apomorphine effects in rats.

When interpreting the data of table 3 it should be emphasized that they all apply to a situation which exists 2–3 hours after intraperitoneal administration of the neuroleptics to mice. A strictly quantitative comparison between any two drugs of this series is therefore unwarranted on the basis of the results presented in this paper

As mentioned in the introduction inhibition of amphetamine-induced stereotyped behaviour in rats is widely used as a selective screening test for neuroleptic activity In our experience the classification of neuroleptics presented in this paper is not much different from that obtained with the amphetamine antagonism test in rats, with one striking exception i.e. reserpine. The drug does not inhibit the effect of amphetamine. In contrast, reserpine accentuates the excitatory effects of amphetamine both in degree and rate of onset (SCHEEL-KRÜGER 1971)

Neuroleptics (except those of the reserpine type) are considered to be blockers of central dopamine receptors, and the inhibition of stereotypies induced by methylphenidate in mice or amphetamine (and apomorphine) in rats is probably a pharmacological index of such activity (JANSSEN & ALLEWIJN 1968) As the methylphenidate antagonism test described in this paper is easy and cheap to perform we consider the method to be very suitable for large scale screening of neuroleptic compounds.

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From the Zoophysiological Laboratory, Department of Zoology
University of Turku, Turku, Finland

Postnatal Development of the Effects of Alcohol and of the Induced Tolerance to Alcohol in Mice

By

Kari Y. H. Lagerspetz

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Abstract. The effects of single and repeated intraperitoneally injected doses of ethyl alcohol on selected reflex responses and open field behaviour of mice aged from 2 to 46 days were studied. The effect of alcohol came on very rapidly in the youngest age groups, the maximum effect occurring already 3 minutes after the alcohol injection. The effect was smaller in 20 days old animals than in 8, 14, or 46 days old mice. Significant growth reductions were found in 14 days old mice which had received alcohol daily for 6 days, and in 46 days old mice which had received alcohol daily for 8 or 18 days. Increased tolerance to alcohol was found in 28 days old and in 46 days old animals after 8 daily injections, but not in 20 days old animals after 6 injections or in the younger age groups. The earliest age at which the induced tolerance to alcohol was found to develop corresponds to the age of maturation of the noradrenergic brain mechanisms, as reflected in the attainment of the adult noradrenaline level in the brain.

Key-words: Ethyl alcohol – postnatal development – induced tolerance – reflex responses – open field behaviour – mouse.

There are few studies on the effects of alcohol in young animals. VOLLMER (1931) found that young mice are more tolerant to alcohol than the adults. In the experiments of CHESLER et al. (1942), the LD 50 after an intraperitoneal injection of alcohol was approximately 5 mg/g for adult and 8 mg/g for newborn rats. HOLLSTEDT & RYDBERG (1970) studied the ethanol elimination in rats aged from 10 to 60 days. The highest rates of ethanol elimination and ethanol disappearance from the blood were found at the ages of 25 to 40 days.

In human infants, alcohol elimination rates as high as 3.5 to 15 times those found in adults have been reported by VERRON (1966). Acute alcohol withdrawal illness was found in newborn delivered by mothers in delirium tremens (NICHOLS 1967). Repeated administration of alcohol has been

quently reported to retard the growth of young animals (for a review see WALLGREN & BARRY 1970 pp 482–489).

In order to gain further information about the effects of alcohol at various stages of the postnatal development, the neurological and behavioural effects of low doses of alcohol were studied in mice aged 2, 8, 14, 20, 28 and 46 days using the reflexological tests described by FOX (1964) and the open field behaviour test. As little is known about the mechanisms involved in the induced tolerance to alcohol, the effect of repeated alcohol administration in inducing tolerance was studied in different age groups, and hence at various levels of development of the CNS mechanisms. The results were compared with the data on the physiological and neurochemical development in young animals.

Material and methods

The test animals were young NMRI mice, which up to 20 days of age had remained with their mothers, and later put into groups in cages where water and food was available *ad libitum*. In each age group, 10 control and 10 test animals were selected by splitting 2 or 3 litters into two groups. The older age groups consisted of 5 male and 5 female mouse pups.

The doses of alcohol used were chosen on the basis of pilot experiments. Ethyl alcohol was injected intraperitoneally in saline solution (10 or 20 per cent w/v) into the animals at dose levels of 1 mg/g (2 days old), 1.5 mg/g (2 and 8 days old), and 2 mg/g (8 days old and older animals). An equivalent amount of 0.9 per cent saline was injected into the control animals.

The 7 reflexological tests used were selected from those described by FOX (1964). The strength of the response was graded to full response (+), weak response (±), and to absence of response (−). In addition, the open-field test (LAGERSPETZ 1972, ERICS-

Table I

Reflexological and behavioural tests used to assess the effects of alcohol in different age groups. + test used, − test not used.

	Age						
	2	8	14	20	28	46	days
1. Righting reflex (RR)	+	+	+	+	+	+	
2. Fore limb placing response (FPR)	+	+	+	+	+	+	
3. Hind limb placing response (HPR)	−	+	−	−	−	−	
4. Fore leg grasp reflex (FGR)	−	+	+	+	+	+	
5. Hind leg grasp reflex (HGR)	−	+	+	+	+	+	
6. Cliff drop aversion (CDA)	−	+	+	+	+	+	
7. Holding on a bar (HB)	−	−	+	+	+	+	
8. Open-field ambulation (OFA)	−	−	−	+	+	+	

son & Wallgren 1967) was used, with ambulation as the only behavioural measure. The tests given to animals belonging to different age groups are listed in table 1. Tests were given before, and at different intervals after the injections, usually 3 and 20 minutes after the injection.

In order to study the development of induced tolerance, animals were injected daily with the corresponding dose of alcohol until they reached the age of the next older age group. Then their response to alcohol was tested and the results compared with those obtained from the animals receiving their first dose of alcohol.

Results

Mice aged 2 to 14 days

The results of the reflexological tests are given in table 2. In all the control groups, full responses were always found after saline injection. Hence the control data are not included in the table.

The onset of the effect of alcohol is very rapid in young animals, the maximum effect being found already at 3 minutes after intraperitoneal alcohol injection in 2 and 8 days old mice. The effect is usually over in 30 minutes or less.

A comparison of the effects of 1.5 mg/g alcohol in 2 and 8 days old mice and of 2 mg/g alcohol in 8 and 14 days old mice shows that the sensitivity of the responses studied to the same doses of alcohol increases from 2 to 8 days, but then decreases again from 8 to 14 days. In the 5 tests (RR, FPR, FGR, HGR, CDA), given both to 8 and 14 days old animals, the 8 days old animals after the injection lost their reflex responses in 42 cases, and retained them in 80 cases. The corresponding values for the 14 days old were 14 and 134 respectively. When evaluated with the chi square method, the difference is significant at the level of $P < 0.0014$ (chi square = 23.9).

The repeated alcohol injections (1 mg/g) did not affect the growth of 2 to 8 days old mice. The weight of the animals receiving the alcohol injections as well as of their saline controls increased on an average by 3.0 g from the age of 2 to the age of 8 days, the standard errors being 0.1 and 0.2 g, respectively.

However, between 8 and 14 days of age the growth was significantly ($P < 0.02$) depressed by repeated alcohol administration (1.5 mg/g), since the increase in weight was on an average 2.6 ± 0.18 g for the animals receiving alcohol daily and 3.1 ± 0.06 g for the controls respectively.

The test results for the animals which had received repeated daily alcohol injections were not markedly different and, at least, not better than those found in animals which had received a single injection only. Thus, the results give no evidence for the development of induced tolerance to alcohol at the early ages studied.

Table 2

The occurrence and strength of different reflexological responses (see table 1) in mice aged 2, 8, and 14 days before and after the intraperitoneal injection of alcohol in the doses indicated. The animals used in the experiments in the right hand columns in the age groups 8 and 14 days received 6 daily injections of alcohol before the days they were tested. Full response is indicated with + weak response with ±, and no response with –

2 days

	1.5 mg/g (two groups)																	
min.	0			3			6			9			12			15		
	+	±	–	+	±	–	+	±	–	+	±	–	+	±	–	+	±	–
1 RR	10			5	5		5	5		9	1		7	3		10		
2. FPR	10			10			10			10			10			10		
1. RR	10			5	5		5	5		7	3		8	2		10		
2. FPR	10			10			10			10			9	1		10		

8 days

	1.5 mg/g											
min.	0			3			20			30		
	+	±	–	+	±	–	+	±	–	+	±	–
1. RR	10			1	5	4	9	1		10		
2. FPR	10			10			10			10		
3. HPR	10			9		1	9		1	10		
4. FGR	10			10			10			10		
5. HOR	10			8		2	9	1		10		
6. CDA	10			10			9	1		10		

	6 × 1 mg/g + 1.5 mg/g														
min.	0			3			20			30			40		
	+	±	–	+	±	–	+	±	–	+	±	–	+	±	–
1. RR	10			2	6	2	7	3		7	3		10		
2. FPR	10			10			10			10			10		
3. HPR	10			9		1	10			10			10		
4. FGR	10			10			10			10			10		
5. HOR	10			3	2	5	10			10			10		
6. CDA	10			9	1		10			10			10		

	2 mg/g											
min.	0			3			10			20		
	+	±	−	+	±	−	+	±	−	+	±	−
1. RR	10					10	1	5	4	7	3	
2. FPR	10			10			10			10		
3. HPR	10			10			10			10		
4. PGR	10			3	4	3	4	3	3	10		
5. HGR	10					10		4	6	6	4	
6. CDA	10			2	3	5	7	2	1	10		

14 days	2 mg/g												6 × 1.5 mg/g + 2 mg/g															
min.	0			3			20			30			0			3			20			40			50			
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	
1. RR	10			9	1		10			10			10			7	3		10			10			10			
2. FPR	10			8	1	1	10			10			10			10			10			10			10			
4. FGR	10			10			10			10			10			9		1	10			10			10			
5 HGR	10			1		9	10			10			10			6			10			10			10			
6. CDA	10			6		4	10			10			10			4	4	2	8	2		9	1		10			
7 HB	10				1	9	7	3		10			10					9		9	1	3	7		10			

Table 3

The occurrence and strength of different reflexological responses (see table 1) in mice aged 20, 28, and 46 days, before and after the intraperitoneal injection of 2 mg/g alcohol. The animals used in the experiments in the right hand column received 6, 8, or 18 daily injections of alcohol before the day on which they were tested. Other explanations as in table 2.

20 days	2 mg/g									7 × 2 mg/g								
min.	0			3			20			0			3			20		
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
1. RR	10			10			10			10			10			10		
2. FPR	10			10			10			10			10			10		
4 FGR	10			10			10			10			10			10		
5 HGR	10			8		2	10			10			9		1	10		
6. CDA	10			10			10			10			10			10		
7 HB	10				8	2	10			10			6		4	10		

28 days	2 mg/g									9 × 2 mg/g								
min.	0			3			20			0			3			20		
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
1. RR	10			10			10			10			10			10		
2. FPR	10			10			10			10			10			10		
4 FGR	10			10			10			10			10			10		
5 HGR	10			10			10			10			10			10		
6. CDA	10			3		7	10			10			10			10		
7 HB	10					10	10			10			5	4	1	10		

	8 × 0.9 % NaCl + 2 mg/g									9 × 2 mg/g								
min.	0			3			20			0			3			20		
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
1 RR	10			10			10			10			10			10		
2. FPR	10			4	5	1	10			10			9	1		10		
4. FGR	10			7	2	1	10			10			10			10		
5. HGR	10					10	7		3	10			2	3	5	7	3	
6. CDA	10			6		4	10			10			10			10		
7 HB	10			1	4	5	10			10			10			10		

46 days	2 mg/g									19 × 2 mg/g								
min.	0			3			20			0			3			20		
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
1. RR	10			10			10			10			10			10		
2. FPR	10			9		1	10			10			10			10		
4 FGR	10			9		1	10			10			10			10		
5 HGR	10			3	6	1	10			10			10			10		
6. CDA	10			8	1	1	10			10			10			10		
7 HB	10				6	4	10			10			4	2	4	10		

	8 × 0.9 % NaCl + 2 mg/g									9 × 2 mg/g								
min.	0			3			20			0			3			20		
	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−	+	±	−
1. RR	10			9	1		10			10			10			10		
2. FPR	10			7	2	1	10			10			10			10		
4. FGR	10			6	2	2	10			10			10			10		
5. HGR	10				1	9	5	5		10			2	5	3	7	3	
6. CDA	10			2		8	10			10			7	2	1	10		
7 HB	10			1	1	8	10			10			5	2	3	10		

Mice aged 20 to 46 days

The results of the reflexological tests are given in table 3 and those of the open-field ambulation measurements in table 4 Full reflex responses were always found after saline injection, and hence the control data are not included in table 3

The onset of the effect of alcohol is still very rapid in the age groups studied. When compared with the data for 14 days old animals, the sensitivity to the same dose of alcohol (2 mg/g) seems to be further decreased in 20 days old animals. After this age, the sensitivity apparently increases again, at least between the ages of 28 and 46 days.

Repeated alcohol injections did not significantly affect the growth of 14 to 20 days old mice. During this interval the increase in body weight was on an average 2.6 ± 0 12 g for those given repeated injections, and 2.7 ± 0.18 g for the saline controls. Growth differences were also not significant in the 20 to 28 days old mice, with weight increases of 4 4 ± 0.26 g and 4.1 ± 0 46 g for the two groups which had received repeated alcohol injections, and 5 1 ± 0.39 g and 5 1 ± 0 40 g for the two control groups, respectively

However a definite impairment of growth ($P < 0\ 05$) occurred during the longer interval of repeated alcohol injections, i. e., from 28 to 46 days of age. The increase in body weight was now 4.9 ± 0 65 g or 29 / of the weight at the beginning of the experiment for the experimental animals and 7 45 ± 0 91 g or 50 / for the controls.

A smaller but significant ($P < 0\ 05$) reduction in growth also occurred in those animals which had received alcohol from 38 to 46 days of age. The increases in the body weight were 0 85 ± 0 18 g or 4 / for the experimental animals and 2.34 ± 0.51 g or 12 / for the controls.

At the age of 28 days, the reflexological data obtained 3 minutes after the injection showed definite differences in the effects of alcohol between the experimental mice which had repeatedly received alcohol and the control mice which have had been given only one injection. The controls in the first experiment had altogether lost 17 responses and gave 43 full responses, and

Table 4

The effects of single and repeated alcohol and saline injections on the open-field ambulation values in mice aged 20, 28, and 46 days. The mean increases or decreases are calculated in per cent from the pre-injection values. Ten animals in each group.

Age in days	Number of alcohol injections (2 mg/g)	Number of saline injections	Increase (+) or decrease (−) in mean ambulation values in per cent after the last injection 4 min.	21 min.
20	1	–	+114	−30
20	–	1	−35	−74
20	7	–	+95	−22
20	–	7	−43	−63
28	1	–	+122	+22
28	–	1	−27	−47
28	9	–	+67	−44
28	–	9	−37	−55
28	9	–	+114	+11
28	1	8	+418	+77
46	1	–	+86	−32
46	–	1	−36	−56
46	19	–	+141	−5
46	–	19	−33	−47
46	9	–	+105	−18
46	1	8	+77	−75

in the second experiment had lost 21 and gave 28 full responses. The experimental animals, again, had in the first study lost 1 response only and gave 55 positive responses, while in the second experiment the corresponding figures were 4 and 54 respectively Statistical evaluation of these data with the chi-square test showed that in the first experiment the difference between the experimental and control animals was significant at the level of $P < 0\ 001$ (chi square = 151 6) and in the second experiment also at the level of $P < 0\ 001$ (chi square = 14 4) On the other hand, the reflexological test performance of animals aged 46 days with previous alcohol administration for 18 days was not significantly different from the performance of their controls (chi square = 1 6), when the statistical significance was evaluated as mentioned above In those 46 days old animals which had received alcohol for 8 days only the performance in the reflexological tests after alcohol administration was significantly ($P < 0\ 001$ chi square = 11.2) better than in their controls. These results show that increased tolerance to alcohol was induced by repeated alcohol injections between the ages of 20 and 28 days

as well as between the ages of 38 and 46 days, but not between the ages of 14 and 20 days or between the ages of 28 and 46 days.

The open-field ambulation data, given in table 4 show that the ambulation values always decreased after the saline injection in successive measurements, as compared to the pre-injection values. This was also the case in the successive control experiments of LAGERSPETZ (1972) and ERIKSSON & WALLGREN (1967). The decreases from the pre-injection values ranged from 27 to 43 per cent in the second presentation of the open-field test to young mice, and from 47 to 74 per cent in the third presentation.

In contrast to this, alcohol (2 mg/g) in young mice always increased the ambulation values well above the pre-injection level as found in adult mice (LAGERSPETZ 1972). This response was definite after the injection in the first open-field test. The values obtained a range from 77 to 418 per cent increase, and showed no indication of changes in the sensitivity to alcohol during the period studied.

The increases caused by alcohol in the ambulation values were larger in 28 days old animals which had received alcohol 8 times previously than in the animals of the same age which had either received no previous injections (Experiment 1) or received saline injections (Experiment 2) In the first experiment the mean increase in ambulation values was 46.8 ± 18 4 for experimental animals and 67 8 ± 15 1 for the controls, the difference not being statistically significant (*t* = 1 07). In the second experiment, the corresponding increases in ambulation values were 25 1 ± 5 6 and 53.5 ± 7.3 for the experimental animals and controls, respectively This difference was significant at the level of $P < 0.01$ (t=3 10). No significant differences were found in the corresponding values for 20 days old animals (t=0.80) and 46 days old animals, whether they had received alcohol for 18 or for 8 days (t=1.74 t=1 41 respectively) These results are generally in agreement with the data gained from the reflexological tests, which also indicate that the development of induced tolerance to alcohol occurs between 20 and 28 days, but not between 14 and 20 and 28 and 46 days of age, respectively How ever reflexological tests indicated that tolerance to alcohol had developed between 38 and 46 days of age.

Discussion

Mice aged 2 days were less sensitive to the effect of alcohol than the 8 days old animals. However this result is based on observations of the righting reflex only and the possible differences in the time elapsed from feeding have not been taken into account.

The effect of alcohol was *smaller in 20 days old* than in 8, 14 or 46 days old animals As HOLLSTEDT & RYDBERG (1970) found a maximum of

alcohol elimination rate at the ages of 25 to 40 days in rats, it is possible that the minimum alcohol sensitivity obtained here at 20 days of age depends on a high alcohol elimination rate at this age. The ethanol-oxidizing capacity of postnatal rat liver slices as well as the alcohol dehydrogenase activity of liver homogenates develop to the adult level between the ages of 13 and 18 to 20 days (RÄIHÄ *et al.* 1967 RÄIHÄ & PIKKARAINEN 1971). The decrease which occurs in the alcohol elimination rate in the older age groups is, as yet, unexplained (HOLLSTEDT & RYDBERG 1970), even when the changes in the liver size are taken into account, but it may be connected with the general decrease of energy metabolism with increasing body size.

Both the reflexological tests and the open field ambulation values showed that increased tolerance to alcohol was induced in mice aged from 20 to 28 days. In contrast, no induced tolerance was found to develop between the ages of 28 and 46 days. However during the latter period the growth of the animals was definitely impaired by long-term repeated alcohol administration, and the simultaneous impairment of the physical condition of the animals may account for the relatively high alcohol sensitivity of the animals which had repeated alcohol injections. A shorter period of repeated alcohol administration between the ages 38 and 46 days impaired the growth less, and resulted in an increased tolerance to alcohol. Significant retardation of growth was also found after daily alcohol administration in mice between the ages of 8 and 14 days, as well as between 38 and 46 days, and a smaller and not significant depression of growth between the ages of 20 and 28 days.

These effects are probably due to a reduced food intake or other depression of the utilization of calories for growth in the animals receiving alcohol daily (SCHEIG & KLATSKIN 1966 for a review see WALLGREN & BARRY 1970, pp. 482–489)

During the age intervals from 2 to 8, from 8 to 14 and from 14 to 20 days there are no indications of development of induced tolerance to alcohol. This absence of tolerance induction cannot be attributed to an impairment of growth, since no significant effects of repeated alcohol administration on growth were found during the age intervals of 2 to 8 and 14 to 20 days, respectively with the dose used. It may be argued that the dose levels used were too low or that too few injections have been given in order to induce tolerance. However the effects of the injections were definite, and even greater in the younger age groups, while the dose used for 14 to 20 days old animals was the same as that which induced increased tolerance in 20 to 28 days old animals. Moreover the number of injections, 6 or 8, may not be sufficient to account for the absence of induced tolerance in 20 days old animals. A more plausible explanation is that the mechanism required for the development of induced tolerance is not fully mature before the age of 20 days in mice.

Increase in the rate of alcohol metabolism could be a possible mechanism of induced tolerance to alcohol. RÄIHÄ & PIKKARAINEN (1971) studied young rats up to the age of 12 days and found no increase in alcohol dehydrogenase activity in liver homogenates of animals to which daily alcohol doses of 2 mg/g had been given. The evidence concerning increased alcohol metabolism in induced alcohol tolerance in adult animals is controversial. In any case, and independent of this, an altered resistance of the central nervous system is considered to be involved in the development of induced tolerance to alcohol (WALLGREN & BARRY 1970 p. 502). Therefore, it would be interesting to compare the postnatal development of the mechanism of induced alcohol tolerance with the development of the central nervous mechanisms in mice.

The postnatal development of the brain mechanisms in mice is fairly well-known. The development of adult-like reflex responses is completed at 13 to 16 days of age (FOX 1964). The electrocorticogram of the mouse attains maturity at the age of 16 to 17 days (KOBAYASHI & HIMWICH 1962) and the histology of the cortex at 15 to 17 days of age (KOBAYASHI *et al.* 1963). The adult 5-hydroxytryptamine level of the brain is attained at the age of 15 days in mice (AGRAWAL *et al.* 1968). All these important mechanisms are reasonably well developed already during the age interval from 14 to 20 days, during which, however, no induction of induced tolerance could be demonstrated. On the other hand, adult noradrenaline level in the brain is attained at the age of 25 to 30 days (AGRAWAL *et al.* 1968), while the concentration of acetylcholine (HIRSIMÄKI & LAGERSPETZ, unpublished results), and especially that of dopamine (AGRAWAL *et al.* l. c.) continues to increase until the adult stage in mice. It is therefore tempting to assume, that the maturation of the noradrenergic brain mechanisms is important for the development of induced tolerance to alcohol in mice, especially as alcohol (2 mg/g) is known to cause a direct or indirect specific activation of the central noradrenergic neurones in the rat (CORRODI *et al.* 1966).

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From the Zoophysiological Laboratory Department of Zoology
University of Turku, Turku, Finland

Diurnal Variation in the Effects of Alcohol and in the Brain 5-Hydroxytryptamine Metabolism in Mice

By

Kari Y H Lagerspetz

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Abstract. Adult male mice show diurnal variation in their performance in the tilted-plane test and in their open field behaviour Maximum sliding angle and minimum open field ambulation values were found at 04.00 hrs, minimum sliding angle and maximum open field ambulation values t 20.00 hrs. Diurnal variation was also found in the effects of ethyl lcohol. Its effect on the sliding angle values was greatest at 20.00 hrs and at 12.00 hrs, and on the open field ambulation at 16.00 hrs. The effects of alcohol were thus more pronounced when the activity level of the animals was high. N clear diurnal variation was found in the brain levels of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) The brain level of 5-HIAA showed trend to a negative correlation ($r = -0.45$) with the open field ambulation scores recorded at the same time of the day The results indicate that the adrenergic and/or serotonergic brain mechanisms may be important in mediating the effects of alcohol.

Key-words: Diurnal variation – ethyl alcohol – tilted plane test – open field behaviour – 5-hydroxytryptamine – 5-hydroxyindole acetic acid – mouse.

There are remarkably few studies concerning the diurnal variation in the effects of alcohol. Haus & Halberg (1959) found that the lethal effect of 8 mg/g ethyl alcohol in mice showed a diurnal maximum at 20 00 hours (8 p.m.) at which time they also recorded the highest body temperatures in their mice. Wilson (1970) reported that the taste thresholds of alcohol in humans are at their lowest at 18.00 hours in the evening and reach a maximum at 06.00 hours. Eriksson (1971) in a recent study showed a clear diurnal rhythm in the voluntary alcohol uptake in rats the maxima of the drinking of ethanol occurred during the dark phase of the illumination cycle, during evening hours and at 04 00 hours.

The present study was undertaken in order to find out if there is a diurnal rhythm in the effects of alcohol at pharmacological dose levels in laboratory mice. The effects of alcohol were assessed by the tilted-plane test (ARVOLA et al. 1958) and by the open field test (ERIKSSON & WALLGREN 1967), now adapted for mice. An attempt was made to correlate the findings with the diurnal variations found in the 5-hydroxytryptamine (5-HT) and 5-hydroxyindole acetic acid (5 HIAA) levels in the brain.

Material and methods

Test animals. Male albino mice of the NMRI strain aged 3.5 to 4.5 months were used as experimental animals. They were housed in groups in cages where food and water was available *ad libitum.* The animals were subjected to artificial light from 06.00 hrs to 22.00 hrs and kept in the dark during the night.

Tilted plane test. The apparatus used was essentially similar to that described by ARVOLA et al. (1958). It consisted of a plywood plane which could be mechanically tilted at a variable speed and stopped in the desired position. It was driven by a variable-speed electric laboratory mixer motor. The animal was placed on the plywood surface with its head pointing in the upward direction and the plane tilted from the horizontal position until the animal began to slide down the surface. Then the tilting of the plane was immediately stopped by disconnecting the gearing to the driving motor and the angle at which the animal slided down was recorded. Three to five measurements were always done, and their results averaged to give the sliding angle for that animal in the test.

Some features of the method were studied in preliminary experiments. The effect of the speed on which the plane was tilted was first studied. Twenty male mice were tested, each with five measurements using the following tilting speeds: 3, 6, 8, and 10 °/sec. The only significant differences (Student's t-test) found in the results were between the highest speed (10°/sec.) and the two lowest speeds (3 and 6 °/sec.). The sliding angle for the higher speed was significantly ($P < 0.05$) higher in both cases. In all further tests, the tilting speed of 10°/sec. was used.

In another experiment, the effects of different doses of alcohol on the performance in the tilted-plane test were studied. Ethyl alcohol was injected intraperitoneally as solutions made in 0.9 % saline containing 10 % (w/v) alcohol (for the dose 1 mg/g body weight) or 20 % alcohol (w/v) (for the doses 2 and 3 mg/g). Three groups each of 10 male animals were used. They were tested on the tilted-plane before the alcohol injection, and 20, 40, 60 as well as 80 minutes after the injection in order to assess the time for the maximum effect. All experiments were done between 10.00 and 14.00 hrs. The mean results are given in table 1 and in fig. 1.

In this experiment, the dose level 2 mg/g was the lowest which gave significant response. It was at maximum at 40 minutes after the injection when the dose 2 mg/g was given, and 20 minutes after the injection for the higher dose, 3 mg/g. The dose-response relationship was fairly linear in the dose range used (fig. 1), and in good agreement with the results of DAMSCHMAND et al. (1967). The dose 2 mg/g was chosen for the further experiments, since it was assumed 1) that the effects of the lower dose levels would be more comparable to the intoxicating effects in man, and 2) that the possible diurnal variation would be more easily seen when the effects of the dose

Table 1

The effect of different doses of alcohol on the mean sliding angle of mice in the tilted-plane test. The significances of the differences from the control values are given in brackets (n. s. = not significant, Student's t-test).

	Control (before inj.)	After the injection 20 min.	P <	40 min.	P <	60 min.	P <	80 min.	P <
1 mg/g	48.0	44.9	(n. s.)	46.9	(n. s.)	47.8	(n. s.)	47.8	(n. s.)
2 mg/g	45.5	38.6	(0.005)	37.1	(0.001)	39.7	(0.025)	42.5	(n. s.)
3 mg/g	54.6	38.6	(0.001)	42.7	(0.001)	44.8	(0.001)	47.6	(0.001)

would be most effectively influenced by the controlling mechanisms involved. In addition, this dose level was obviously within the linear range of the dose-response relationship.

Open-field test. ERIKSSON & WALLGREN (1967) have studied the behaviour of rats under the influence of alcohol in the open-field situation. They found that the lowest dose (0.75 mg/g) which affected the behaviour significantly increased the time during which the animals were motionless, decreased the non-locomotor activity and depressed preening in rats. In this study advantage was taken of ARVOLA's (1960) finding that alcohol in doses of 1 to 2 mg/g increased spontaneous ambulation in mice. A circular white-painted arena with a diameter of 40 cm, divided by painted circular and radial lines in to 19 partitions of approximately the same area, was used as the open-field. No sound stimulation was used, but the arena was brightly lit, the intensity of illumination being about 1800 lux at the open field level.

During the two-minute session in the open-field, the following behavioural measures were recorded with the aid of hand-tallies: 1) the number of the partitions the animal

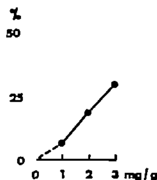


Fig. 1. Maximum reduction in the mean sliding angle in the tilted-plane tests by different doses of alcohol in mice (in per cent of the control values obtai alcohol injection).

Table 2

The effect of 1 mg/g alcohol on some behavioural measures in the open-field test. The significances of the difference from the control values are given in brackets (n. s. = not significant, Student's t-test)

	Control (before inj.)	After the injection 30 min.	P <	60 min.	P <
Ambulation	74.4	113.1	(0.05)	72.0	(n. s.)
Rearing	11.6	12.9	(n. s.)	12.0	(n. s.)
Preening	0.5	0.6		0.4	
Defaecation	1.2	0.5		0.2	

entered (ambulation) 2) the number of times it rose on its hind-legs (rearing), 3) the number of preening episodes, and 4) the number of stools produced during the session.

In a preliminary experiment, 10 male mice were given an open-field test session before the injection of 1 mg/g alcohol (as 10 % *(w/v) solution in 0.9 %* NaCl), and further test sessions 30 and 60 minutes after the intraperitoneal injection. The results of this experiment are given in table 2.

A statistically significant effect was only obtained in the ambulation in the open-field. In mice, rearing was not affected by alcohol at the dose level used, and the frequencies of preening and defaecation were too low to be analysed. The dose of 1 mg/g was considered suitable for the present investigation, since it obviously was close to the lower dose limit at which significant response could still be elicited.

Determination of 5-HT and 5-HIAA levels. After decapitation, the brain was rapidly dissected out, and the forebrain (weight about 250 mg) and the brain stem, including the hypothalamus, midbrain, pons and medulla, but not the cerebellar hemispheres (weight about 140 mg) separated, deep-frozen, and later homogenized in ice-cold acidified n-butanol. The determination of 5-HT and 5-HIAA was made according to the method of Raab (1970) and the fluorescence measurements with an Aminco-Bowman spectrofluorometer

Results

Diurnal variation in the test performance

The open-field and tilted-plane tests were given in that order to 8 groups each of 6 male mice. The groups being matched for age and weight. The tests were made on two of the groups beginning at 12.00 hrs and for the other two at 24 00 hrs, and on the remaining 4 groups beginning at 04 00 08.00, 16.00, and 20 00 hrs, respectively The mean sliding angle and ambulation values are given in fig. 2.

Diurnal variations in the performance of mice in the two tests are evident. Maximum mean values for the sliding angle was found at 4.00 hrs, minimum

at 20.00 hrs, while the picture is reversed for the ambulation values of the same animals, which show a maximum at 20.00 hrs and a minimum at 04 00 hrs. The difference between the mean ambulation values at these times is statistically significant ($P < 0.05$), when evaluated with Student s t test, while the difference in the sliding angle values is not. However the difference between the mean sliding angle values recorded at 20 00 hrs and at 24 00 hrs is statistically significant ($P < 0.01$), owing to the larger material (12 animals) tested at 24.00 hrs.

Diurnal variation in the effect of alcohol in the tilted plane test Three groups each of 10 male mice were matched for age and weight. The effect of 2 mg/g ethyl alcohol on the performance of these animals in the tilted-plane test was measured twice in each group at an interval of 2.5 days. Thus, for the first group the alcohol was injected at 04 00 hrs and at 16.00 hrs, for the second group at 08.00 hrs and 20 00 hrs, and for the third group at 12.00 hrs and 24.00 hrs. The sliding angle of the animals was measured as described above, before and 20 40 and 60 minutes after the injection. The results are given in fig. 3

When testing the statistical significance, the differences from the pre injection sliding angles were found to be significantly ($P < 0\ 01$) larger at 20.00 hrs and 12.00 hrs than at 08 00 hrs. In this experiment, the greatest effect of alcohol was thus found at 20 00 hrs and 12.00 hrs, the values for these times not being significantly different.

The maximum reduction in the mean sliding angles caused by alcohol was 7 / at 08.00 hrs and 13 / at 12.00 hrs. When compared with the slope of the

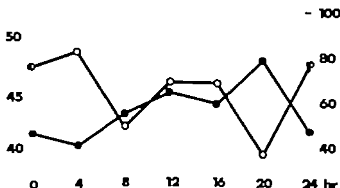


Fig 2. Diurnal variation in the performance of mice in the tilted-plane and open-field tests. Open circles: mean sliding angle in the tilted-plane test (values on the left). Dots: mean ambulation values in the open-field test (values on the right). Di animal groups were used at each time indicated.

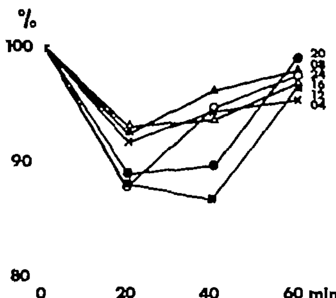


Fig. 3 The effect of 2 mg/g alcohol on the performance in the tilted-plane test at different times of day given as the percentage of the sliding angle from that recorded before the alcohol injection (at 0 min.). Hours indicated at the ends of the graphs.

dose-response line (fig. 1) it appears that the magnitude of diurnal variation corresponds to a dose difference of 0.5 mg/g.

Diurnal variation in the effect of alcohol on the ambulation in the open-field test The open-field behaviour of two groups each of 10 male mice was studied before as well as 30 and 60 minutes after 1) an alcohol injection of 1 mg/g given at 16.00 hrs, 2) an alcohol injection of 1 mg/g given at 04.00 hrs, 3) a saline injection given at 16.00 hrs, and 4) a saline injection given at 04.00 hrs. Thus, all 20 animals received the same treatments. The intervals between the treatments were, at least, three days. Table 3 gives the mean results for this experiment.

These results show that, as compared with the saline injections, alcohol, at the low dose level used, significantly increased ambulation in the open-field test both at 04.00 hrs and at 16.00 hrs, although this effect was significantly higher and lasted longer at 16.00 hrs. It is also interesting that the ambulation values decreased with the three successive presentations of the open field situation to the animals, as found with one-day intervals by ERIKSSON & WALLGREN (1967). This effect was more pronounced in the night-time than in the day-time experiments, but the difference between the ambulation values recorded before the saline injection at 04.00 hrs and one hour after did

Table 3

The effect of saline and 1 mg/g alcohol on the mean ambulation in the open-field test at 04.00 hrs and 16.00 hrs. The P values give the statistical significance level of the difference between the two adjacent means in the next column to the right (n.s. = not significant, Student's t-test)

		Control (before inj.)	P <	After the injection 30 min.	P <	60 min.	P <
04.00 hrs	NaCl	51 45		40.15		33.05	
			n.s.		0 02		n.s.
	alc.	58.30		65.15		36.30	
			n.s.		0.05		0.01
16 00 hrs	alc.	61 75		95.95		64.75	
			n.s.		0.001		0.02
	NaCl	45.90		43.05		41.75	
			n.s.		n.s.		n.s.
04 00 hrs	NaCl	51 45		40.15		33.05	

not reach statistical significance, i.e. ($0 1 > P > 0 05$) The low ambulation values recorded at that time are in agreement with the occurrence of the ambulation minimum in fig. 1

Diurnal variation in 5-HT and 5-HIAA levels in the forebrain and in the brain stem. The animals used for the 5-HT and 5-HIAA determinations were the same 8 groups each of 6 animals, in which the diurnal variation in the test performance was assessed. The animals were sacrificed immediately after the open-field and tilted-plane test performed at 04 00 08 00 12.00, 16 00, 20 00 and 24.00 hrs. The average results of the determinations as well as the standard deviations are given in fig. 4 where the graph showing the diurnal variation in the open-field ambulation in these animal groups is also depicted.

The analysis of variance failed to reveal any significant diurnal variation in the 5-HT and 5-HIAA concentrations. However when the values for the lowest (04 00 hrs) and highest (20.00 hrs) open-field ambulation activities are compared with Student's t-test, the 5-HIAA levels were significantly higher at 04 00 than at 20 00 hrs both in the forebrain and in the brain stem ($P < 0 01$). Significant differences were also found between 5-HT levels at 04 00 hrs and 24 00 hrs both in the forebrain and in the brain stem ($P<0.01$), and between 5-HIAA levels at the same times of day in the forebrain ($P < 0.001$) and in the brain stem ($P < 0 05$)

It is interesting that the 5-HIAA level in the brain stem shows a trend to a negative correlation ($r = -0 43$) with the ambulation values recorded from

the same animals, as can be seen in fig. 4. However, this does not reach the level of statistical significance.

Discussion

The preliminary experiments made in connection with the present study show that the *tilted-plane test* as well as the ambulation test in an open-field situation can be used as criteria for the effect of alcohol in mice. The open-field ambulation is obviously the more sensitive of these two measurements.

Other results of importance for the methodology used in alcohol studies are the significant diurnal variations which are shown by the control values

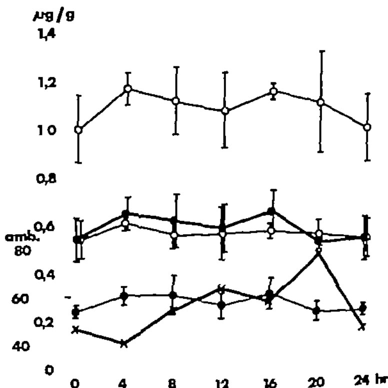


Fig. 4. 5-HT and 5-HIAA levels in the brain stem and in the forebrain of mice at different times of day. At each mean ± S.D. is shown. The mean ambulation scores of the same animals (taken from fig. 2) are also given. The uppermost graph (open circles): 5-HT in brain stem; the lower graph with open circles: 5-HT in the forebrain; the upper graph with black dots: 5-HIAA in the brain stem, the lower graph with black dots: 5-HIAA in the forebrain; the graph with crosses: ambulation values.

of both the measures used, and also the occurrence of significant diurnal variation in the effects of low doses of alcohol on these test performances. The influence of the time of day at which the effects of alcohol are measured in experimental situations should therefore be taken into account when comparisons are made between the results of different experiments.

Regarding the timing of the diurnal variation in the effect of alcohol on the behavioural measurements used, the maximum effect in the tilted-plane test was found during the hours of maximum ambulation, 20.00 hrs and 12.00 hrs while the effect on ambulation was also higher during the period of activity than during the rest period. In the only previous animal study on the diurnal variation in the effects of alcohol, HAUS & HALBERG (1959) found the maximum lethal effect of 8 mg/g alcohol at 20.00 hrs, while this effect was at its minimum during the night hours. Taken together these findings suffice to show that there is a definite diurnal rhythm in the susceptibility to the effects of alcohol in mice.

As there is no general agreement regarding the mechanisms of action of alcohol, it is not easy to point out the physiological mechanism underlying this rhythm. It is well known that there is a diurnal rhythm in the blood glucose level in mice (BULLOUGH 1949) and, on the other hand, that increased blood glucose values have been found to cause a decrease in the effect of alcohol in the tilted-plane test in rats, while lowered blood sugar enhances this effect of alcohol (SAMMALISTO 1962). However the maxima of the blood glucose levels are found at 20.00 hrs and 10.00 hrs and the lowest levels from 02.00 to 06.00 hrs (BULLOUGH 1949 table 1). The highest blood glucose levels thus coincide with the time of the greatest effects of alcohol found in this study and vice versa. It can be concluded that the diurnal rhythm in the blood glucose level and the attenuating effect of high blood glucose levels on the degree of intoxication cannot explain the diurnal rhythm in the susceptibility to alcohol in mice, but rather that this rhythm persists in spite of the antagonistic action exerted by the blood glucose rhythm.

The present finding that alcohol affects the performance in the tilted-plane test more during the period of activity of the animal than during the rest period, may perhaps be explained by the stronger effect of alcohol on the motor activity which also occurs during the active period. The animals are made more restless by alcohol in the daytime and evening than during the night, and this may more quickly initiate sliding from the tilted-plane. Whether the increase of activity and the resulting earlier sliding from the tilted-plane are combined with a reduction of fear caused by alcohol in the experimental situations, cannot be decided on the basis of the present experiments.

The stronger effect of alcohol on ambulation in the open-field during the period of higher basic activity reveals an interesting aspect of the activity rhythm in general when the animal is more active it is also tuned to respond

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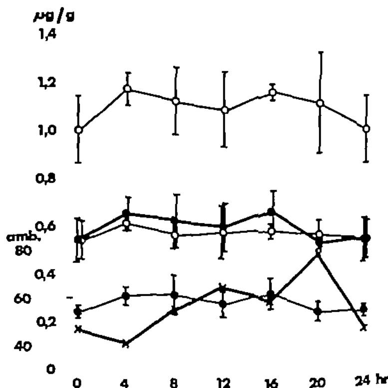


Fig. 4 5-HT and 5-HIAA levels in the brain stem and in the forebrain of mice at different times of day At each mean ± S. D is shown. The mean ambulation scores of the same animals (taken from fig. 2) are also given. The uppermost graph (open circles): 5-HT in brain stem, the lower graph with open circles: 5-HT in the forebrain; the upper graph with black dots: 5-HIAA in the brain stem, the lower graph with black dots: 5-HIAA in the forebrain, the graph with crosses: ambulation values.

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more intensely to activating effects, like that provided by alcohol. This corresponds to the notion of high arousal level.

ALBRECHT *et al.* (1956) found higher 5-HT levels in mouse brain during the morning hours than during the afternoon or evening. However the existence of a diurnal rhythm in the brain 5-HT level in mice has not always been confirmed (SAELENS *et al.* 1968 SOFIA & SALAMA 1970). As the results of the present study also show the reproducibility of the diurnal 5-HT variation is obviously poor but a nightly maximum of the activity of 5-HT metabolism, corresponding to the nightly minimum of motor activity in mice can possibly be discerned

In view of the suggested role of serotonergic mechanisms in the process of sleep (JOUVET 1969) it is not surprising to find a trend to a negative correlation between the open-field ambulation and the level of 5-HT metabolite 5-HIAA in the brain stem, as found in the present study (fig. 4) The low 5-HIAA level probably reflects a low rate of 5-HT metabolism and a low activity of the serotonergic neurones in the brain stem, which would then be associated with a high motor activity as shown by the ambulation scores, high alcohol susceptibility and high arousal level.

However it must be pointed out that the 5-HT-ergic mechanisms are possibly not the only brain mechanisms with a diurnal rhythm and which affect the arousal level. The depletion of noradrenaline (NA) in the brains of mice after inhibition of its synthesis by 200 mg/kg disulfiram was 45 / greater at 16 00 hrs than at 05 00 hrs (JULKU *et al.* 1970) This indicates a greater overall activity level of the noradrenergic neurones in the brain during the active period than during the rest period. In the experiments of SAELENS *et al.* (1968) the increased brain NA and 5 HT levels in mice were followed by an increase in the daily activity maximum and the depletion of brain NA decreased night-time activity while depletion of brain 5-HT was without any effect. These results suggest that the daily activity maximum in mice is dependent on the function of the noradrenergic systems in the brain.

In rats, the highest histamine and NA levels in the midbrain and in the caudate nucleus and the lowest 5-HT levels in these areas are found at the time when the motor activity and body temperature are maximal in these animals (FRIEDMAN & WALKER 1968) It may well be that a factor involved in the control of the arousal level is the balance of the activities of the chemically different neurone systems in the brain. Alcohol may act by changing the activities of these neurones – if it acts through impairment of the neurone function and thus by decreasing neurone activity then its principal target may well be in the noradrenergic systems, the increased activity of which is correlated with the increased motor activity which is the characteristic effect of low alcohol doses in mice. CORRODI *et al* (1966) demonstrated that the central NA neurones in the rat are specifically activated, directly or indirect

ly 2 to 4 hrs but not 6 hrs after 2 mg/g alcohol These findings may reflect the reciprocal interactions of the NA and serotonergic brain systems which are probably of importance both in the control of activity and in the mediation of the effects of alcohol.

The timing of the diurnal rhythm in the susceptibility to alcohol in mice is probably exogenous in origin, as is the timing of the activity rhythm. The activity rhythm, at least in rats, can be reversed by reversal of the illumination cycle, and the minimum 5 HT levels in the brain are again found during the period of maximal activity and vice versa (DIXIT & BUCKLEY 1967)

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From the Institute of Medical Physiology C, University of Copenhagen,
71 Raadmandsgade, DK 2100 Copenhagen Ø Denmark

Effects of Long-Term Administration of Bendroflumethiazide on Bone Metabolism in the Rat

By

F Schønau Jørgensen and S Pors Nielsen

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Abstract ^{45}Ca was injected subcutaneously in 160 g rats kept in metabolic cages. After an adjustment period of 10 days, bendroflumethiazide was given orally for another 10 days. The concentration of ^{45}Ca and the specific activity in the bones was significantly higher in the thiazide treated animals than in controls. The excretion of hydroxyproline decreased slowly during the thiazide administration period. The results suggest that the breakdown of both bone mineral and bone matrix was reduced during bendroflumethiazide administration. Bone matrix metabolism was not normalized until 7 days after cessation of thiazide administration as judged by the time required for reversion of the hydroxyproline excretion.

Key-words: Bendroflumethiazide – thiazides – calcium – metabolism – bone.

Long-term thiazide administration causes a reduction of renal calcium excretion in man (LAMBERG & KUHLBÄCK 1959 Higgins *et al.* 1964 YENDT *et al.* 1966 PARFITT 1969 YENDT 1970) and in the rat (JØRGENSEN 1971)

Conflicting results have been presented regarding the extrarenal effects of thiazide diuretics on calcium metabolism.

The present experiments were carried out in an attempt to elucidate the effects of long-term thiazide administration on bone metabolism in normal rats. The *results suggest that depression of the renal excretion of calcium* is associated with a decreased bone turnover

Material and Methods

White male Wistar rats (160 g) from Møllegaard Hansens's breeding centre were used in all the experiments. All the rats received 40 µcl ^{45}Ca by subcutaneous injection ($CaCl_2$ in aqueous solution, specific activity 30 µcl/µg, Amersham, England) After a period of 7 days the rats were placed in metabolic cages as previously described (NIELSEN *et al.* 1969- JØRGENSEN 1971) On the 10th day after ^{45}Ca-labelling one

group of rats was given bendroflumethiazide orally while another group served as controls. Bendroflumethiazide (centyl®) was dissolved in water (30 µg/ml) and given in an amount of 30 ml mixed with 20 g of dried food (altromin®) a day. Since the food intake was not less than 15 g/24 hours in any rat, 625-900 µg bendroflumethiazide/24 hours was given. The bendroflumethiazide dose was supra-maximal with regard to the effect on the renal excretion of sodium (KOMURCAN & KATIC 1960) and calcium (JØRGENSEN 1971). The amount of food ingested was measured every 24 hours. After thiazide administration for 10 days all the rats were killed by exsanguination from the aortic bifurcation under light ether anaesthesia (in a non-fasting state). The right clavicle and the right tibia were removed and carefully dissected free of connective tissue. All bone samples were dried to constant weight in a vacuum oven at 65° and 200 mmHg, and ashed by the addition of equal amounts of 70 per cent perchloric acid and 65 per cent nitric acid, and placed in an aluminium block at 210° for 33 hours after slow heating for 3 hours (HATTINGBERG *et al.* 1966). The ashed material was dissolved in 0.1 N hydrochloric acid, after which 2000 µl of quartz redestilled water was added and ^{45}Ca was measured by liquid scintillation counting (Liquid Scintillation Counter Mark II, Nuclear Chicago) using the Triton X 100 system (NADARAJAH *et al.* 1969). 100 µl ^{45}Ca containing solution was mixed with 900 µl H_2O and added to 8 ml of the scintillant. The standard deviation of ^{45}Ca concentration in bone after ashing (8 ashings of the same bone sample) was 1.8 per cent. The bone ash did not interfere with the counting rate of an aqueous standard (NIELSEN, unpublished results). ^{45}Ca in the serum was determined in the same way (100 µl serum plus 900 µl re-destilled water added to 8 ml of the scintillant); no haemolysis was seen in any of the blood samples.

^{40}Ca was determined by atomic absorption (Perkin Elmer Atomic Absorption Spectrophotometer 290 B, acetylene air flame, three slot Boling Burner). One per cent lanthanum oxide was used for the preparation of standards and samples in order to obviate phosphate interference. Hydroxyproline in the urine was measured in two series of experiments in which 45 µg and 90 µg bendroflumethiazide per g food was given. Hydroxyproline was determined by a slight modification of the method of KIVIRIKKO *et al.* (1967).

Results

The mean weights of the rats (mean ± S.D.) in the control and the thiazide groups of the ^{45}Ca experiment were, respectively 161 ± 5 g ($n = 7$) and 157 ± 8 g ($n = 6$) at the beginning of the administration period and 205 ± 6 g and 194 ± 9 g after 10 days ($P > 0.1$). The mean weights of the bones used for analysis were: Tibia 45.8 (3.8) mg and 49.4 (3.6) mg, clavicle 12.2 (0.9) and 12.1 (0.5) (mean (S.E.M.)) in the control and the thiazide group respectively.

The renal excretion of ^{45}Ca was the same on the day before thiazide administration, 21677 (3703) and 21563 (3806) cpm/24 hrs (S.E.M.) in the control and the thiazide group.

The urinary excretion of ^{45}Ca during the thiazide administration is shown on fig. 1. A significantly reduced excretion of ^{45}Ca in the thiazide group

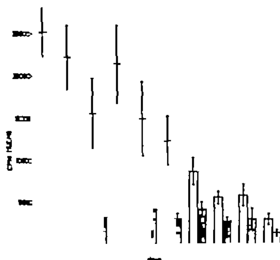


Fig. 1. The effect of bendroflumethiazide on the renal excretion of ^{45}Ca in rats prelabelled 10 days before the collection of urine compared with an untreated control group. The filled columns indicate the thiazide group, the open columns the control group

was seen during the whole administration period. The specific activity was not measured.

The concentration of stable and radioactive calcium in bones after 10 days of thiazide administration is shown in table 1 No difference in ^{40}Ca concentration between the control group and the thiazide group could be detected, but the concentration of ^{45}Ca in the bones and the specific activity was significantly higher in the thiazide group than in the controls.

The serum concentration of ^{40}Ca and ^{45}Ca 10 days after thiazide administration was measured in the same experiment. The mean concentration of ^{40}Ca in the thiazide group and the control group was 2.45 (0 05) and 2.54 (0.02) mM (S.E.M.) respectively ($P > 0\ 1$). The mean concentration of ^{45}Ca was 34702 (1496) and 32886 (1224) cpm × ml^{-1} (S.E.M.) respectively ($P > 0\ 1$)

In seperate experiments done with the same metabolic technique, 7 rats were given 45 μg bendroflumethiazide/g food for 7 days, and 7 other rats were given 90 μg bendroflumethiazide/g food. The pre-administration period lasted for 5 days and the post-administration period 7 days. The urinary hydroxyproline and ^{40}Ca excretion were measured daily and related to the intake of food. A significant fall in hydroxyproline excretion was seen during the last few days of the thiazide administration period, and this was slowly reversed after thiazide administration was stopped. The change in hydroxyproline excretion was most marked with a dose of bendroflume

Table 1

	^{40}Ca, µmol/mg dry bone		^{45}Ca, cpm/mg dry bone		Specific activity $^{45}Ca/^{40}Ca$ (cpm/µmol)	
	clavicl	tibia	clavicle	tibia	clavicle	tibia
Thiazide group (n = 7)	5.75 (0.09)	6.32 (0.11)	18788 (602)	18645 (611)	3265 (73)	2951 (10)
Control group (n = 6)	5.60 (0.08)	6.42 (0.13)	15675 (337)	15220 (474)	2805 (75)	2392 (82)
P^*	> 0.10	> 0.10	< 0.01	< 0.01	< 0.01	< 0.01

Concentration of ^{40}Ca and ^{45}Ca in the clavicle and the tibia of rats after 10 days of bendroflumethiazide (centyl®) administration compared with a non-treated group. Mean values (S.E.M.). ^{45}Ca given by subcutaneous injection 20 days previously

* Students t-test.

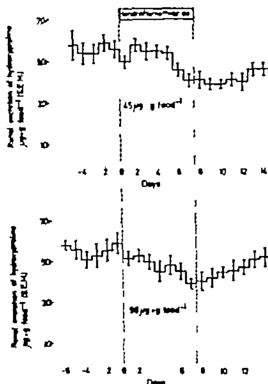


Fig 2. Dose-dependent reduction in renal hydroxyproline excretion. Above: 10 male rats, weight 180 ± 7 g (mean ± S.D.) bendroflumethiazide intake 625–900 μg × 24 hrs⁻¹ Below: 7 male rats, weight 174 ± 5 g (mean ± S.D.) bendroflumethiazide intake 1350–1800 μg × 24 hrs⁻¹. Comparison between basal excretion and the excretion on the 7th day of bendroflumethiazide administration by Student's t-test. $P < 0.005$ for both doses. Comparison between the excretion on the 7th day and on the 14th day $P < 0.05$ for both doses.

thiazide twice the amount given in the radioactive experiment (fig. 2). The calcium excretion pattern which has previously been published (JØRGENSEN 1971) shows a gradual decrease during thiazide administration with minimum excretion values from the 4th day of administration, and a marked temporary rise following cessation of thiazide administration at the same time as the hydroxyproline excretion was normalized.

Discussion

Since the calcium pool labelled in the present experiments is a deep bone pool from which the release of ⁴⁵Ca can be influenced by changes in the

metabolic activity of bone cells (JOHNSTON & DEISS 1966, O'RIORDAN & AURBACH 1968), the results suggest that the net result of prolonged thiazide treatment on bone calcium transfer is an inhibition of bone resorption. Whether this phenomenon is caused by an action of the thiazides directly on bone or whether it is secondary to other effects e. g. to the effect on the kidney, or both, remains to be established.

The suggestion of PARFITT (1969) and KOPPEL *et al.* (1970) that thiazides produce hypercalcemia due to either a stimulating effect on the parathyroid glands or a potentiation effect on the action of the parathyroid hormone is not in agreement with the results of our experiments. A secondary hypersecretion manifested by hyperplasia of the parathyroid glands as found by PICKLEMANN *et al.* (1969) would, however, be expected if thiazides act on bone by inhibiting bone resorption. Previous experiments in this laboratory (JØRGENSEN 1971) have shown that the reduction in renal calcium excretion in rats during thiazide administration is also seen in the absence of the parathyroid and thyroid glands. Therefore, the effect of thiazides on the renal calcium excretion is not likely to be caused primarily by changes in the secretory rate of parathyroid hormone or calcitonin. The temporary rise in renal calcium excretion after the end of the thiazide administration period might be a sign of a hitherto hidden (compensatory) hypersecretion of parathyroid hormone, since it is not seen in parathyroidectomized rats (JØRGENSEN 1971). The unchanged renal hydroxyproline excretion at the beginning of the thiazide administration period might be due to a compensatory hypersecretion of the parathyroid glands, this mechanism being exhausted at the end of the thiazide administration period, making the bone effect overt.

The hydroxyproline excretion pattern in combination with the excretory calcium peak seen after the thiazide administration has stopped, indicates that calcium metabolism is not normalized until 7 days after the thiazide administration has stopped.

Our observation that renal hydroxyproline excretion is depressed by thiazides in the rat is in agreement with the findings in man (YENDT 1970). The observation by BRICKMAN *et al.* (1971) that a reduction in the renal excretion of calcium is not seen in adult patients with hypoparathyroidism, in whom bone turnover is excessively low, supports the hypothesis that the reduction in renal calcium excretion can, at least partially, be explained by a reduction in bone resorption.

The reversal of hydroxyproline excretion towards normal values after cessation of bendroflumethiazide administration is a stronger indication of a bendroflumethiazide-induced depression of bone matrix turnover than the reduction of excretion during the administration period itself since the excretion of hydroxyproline normally decreases moderately with age (FLANA-

GAN & NICHOLS 1969), and also under identical experimental conditions (NIELSEN & JØRGENSEN 1972)

The absence of a reduction of ^{45}Ca or ^{47}Ca concentration in the blood in our experiments is not in disagreement with the hypothesis that the amount of calcium filtered is reduced during thiazide administration, since only minimal (undetectable) decreases in the plasma calcium concentration are necessary to lower the filtration load of calcium sufficiently to depress the renal excretion of ^{45}Ca in the case of an unaltered tubular reabsorption. HARRISON & ROSE (1968) demonstrated that thiazide treatment in some patients is capable of changing a negative calcium balance into a positive direction. Whether the inhibitory effect on bone resorption suggested by our results is long-lasting and can be used in the treatment of patients with an increased bone turnover awaits further studies.

Acknowledgments

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From the Institute of Pharmacology University of Oslo,
Blindern, Oslo 3 Norway

Opposing Anticholinergic and Cardio-depressive Effects of Promazine and Thioridazine in Isolated Rat Atria

By

J. F W Haffner and K. Landmark

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Abstract. Acetylcholine (ACh) reduced both the rate of contraction and the contractile force i isolated rat atria, but these effects were less pronounced after the preparations had been treated with promazine or thioridazine. The anticholinergic activity of the phenothiazines was counteracted by their direct cardiodepressive effects. In electrically stimulated atria treated with promazine 5×10^{-6}M, the anticholinergic effect was dominant, the reduction in contractile force was less than that produced by ACh when acting alone. In the spontaneously beating atria promazine 5×10^{-6}M produced a depression of frequency which balanced the anticholinergic effect. The reduction in the work index produced by ACh was less in the presence of promazine 5×10^{-6}M because of the anticholinergic effect on the contractile force. The cardiodepressive effects of thioridazine 5×10^{-6}M and promazine 10^{-5}M and 2.5×10^{-5}M balanced or exceeded the anticholinergic effects the reductions in cardiac performance were equal to, or greater than those produced by ACh when acting alone. It is suggested that the anticholinergic activity of phenothiazine derivatives is unspecific i.e. not due to receptor blockade, but to general membrane stabilizing effects, which counteract the changes in membrane permeability produced by ACh.

Key-words: Phenothiazines – cardiac anticholinergic effects.

The phenothiazines have been reported to reduce the inhibitory effects of acetylcholine (ACh) on the heart. Thioridazine, for example blocks the effect of vagal stimulation in the dog, and in concentrations of 1 to 2 mg/ completely prevents the inhibitory action of 0 1 to 0.2 mg°/ (ACh) on the isolated rabbit auricles (MADAN 1963). Chlorpromazine has similar effects (COURVOISIER *et al.* 1953). The mechanism underlying these anticholinergic properties has not been elucidated. The phenothiazines may interfere with the cholinergic receptor but it is also possible that the anticholinergic properties of these drugs, which have been classified as membrane stabilizers (LANGSLET 1970· LANDMARK & ØYE 1971) are due to more unspecific effects on the myocardial cell membrane.

In order to determine the nature of the anticholinergic effects of the phenothiazine derivatives, we have studied the action of varying concentrations of promazine (an aliphatic derivative) and of thioridazine (a piperidine derivative) on the dose-response curve to ACh in isolated rat atria. High concentrations of promazine and thioridazine have direct cardiodepressive effects (LANDMARK 1971b) which oppose their anticholinergic effects. In the present experiments we have tried to evaluate the relative importance of these two actions of the phenothiazines.

The effects of the phenothiazines were first studied in electrically stimulated atria in order to determine their influence on the contractile force at a constant frequency. The effects of the same concentrations of the phenothiazines were then studied in spontaneously beating preparations in order to determine their effect on frequency and work index.

Material and Methods

Female, albino Wistar rats (about 200 g) were anaesthetized with ether, the hearts were excised and the atria removed by dissection. The experiments with spontaneously beating atria were performed with "double-atria", i. e. left and right atria dissected out "en block", while the experiments with electrically stimulated preparations were performed with left atria. The atria were tied to an acrylic holder and mounted in an organ bath containing 30 ml modified Ringer solution at 32° and bubbled with 95 % O_2 and 5 % CO_2. The Ringer solution had the following composition (ions in meq/l): Na^+ 143.4, K^+ 5.3, Ca^{++} 5.1, Mg^{++} 2.3, Cl^- 126.4, $H_2PO_4^-$ 2.4, HCO_3^- 25, SO_4^{--} 2.3 and glucose 1.8 mg/ml. pH was 7.4. A preload of 400 mg was put on the preparations and the contractile force was recorded isometrically with a Grass force-displacement transducer (FT03C) connected to a Grass polygraph (Model 7WC12PA). The atria were electrically stimulated through bipolar platinum electrodes at a frequency of 180 per min. by square wave pulses of 0.5 msec. duration, delivered by a Biotronic laboratory stimulator. The voltage used was 3 times the threshold as it was found that otherwise some preparations failed to follow the stimulation after addition of the drugs. The threshold varied from 0.6 to 2.0 V as measured on a Tektronix Dualbeam Oscilloscope, type 502A.

After an equilibration period of 30 min., the effect of ACh in bath concentrations of 5×10^{-8}, 2.5×10^{-7}, 10^{-6}, 5×10^{-6}, 2.5×10^{-5} and 10^{-4} was determined. The bath was completely emptied and refilled between each addition of the compound, and the atria were left for 2–3 min. to equilibrate before the next addition of ACh was made. When the three highest concentrations of acetylcholine were used, it was necessary to empty and refill the bath 2–3 times in order to regain the initial contractile strength and rate.

After a second equilibration period of 20 min., the procedure was repeated in the presence of promazine, thioridazine or atropine, which were added to the organ bath 10 min. before the second series of ACh additions, and subsequently after each wash-out. As high concentrations of phenothiazine derivatives cause a steep decline in contractile force, it was necessary to modify the procedure when the anticholinergic effects of promazine 10^{-5} M and 2.5×10^{-5} M were tested. The second dose-response curve for ACh was therefore limited to four concentrations (10^{-6}, 5×10^{-6}, 2.5×10^{-5}

and 10^{-4}M) in atria treated with promazine 10^{-5}M and to two concentrations (10^{-6} and 5×10^{-6} or 2.5×10^{-6} and 10^{-4}M) in atria treated with promazine 2.5×10^{-5}M.

The sensitivity to ACh varied in individual preparations, but was relatively constant from the first to the second dose-response curve in the same preparation. The maximal effect of ACh also varied, so that all results were converted into per cent of the greatest reduction of contractile force or rat produced by ACh (called 100 %) in the first dose-response curve. This enabled us to compare the alterations in ACh sensitivity in different preparations.

The following parameters were studied.

A. In electrically stimulated atria.

1. The reduction in contractile force produced by ACh before (1st dose-response curve ⊢ in figs. 2 5) and after exposure to atropine, promazine or thioridazine (2nd dose-response curve dotted line in figs. 2 5).
2. The alteration in initial contractile force from the first to the second dose-response curve. As the preparations were washed between each addition of ACh, the initial contractile force varied slightly for the different concentrations of ACh. It was therefore necessary to determine the alteration in the initial contractile force from the first to the second dose-response curve for each concentration of ACh. These alterations were very small in the control group, where no drug was added between the two dose-response curves. The alterations in initial contractile force which occurred after the addition of atropine, promazine or thioridazine, could therefore be considered as the direct effect of these drugs.
3. The combined effect of ACh and atropine, promazine or thioridazine, i.e. the sum of the alteration in contractile force produced by atropine or the phenothiazines, and that produced by ACh in the second dose-response curve (● in figs. 2–5).

The parameters studied are demonstrated in fig. 1.

B. In spontaneously beating double-atria.

1. The reduction in rate of contraction produced by ACh before and after exposure to promazine or thioridazine.
2. The reduction in rate of contraction produced by the phenothiazines (see above; A 2).
3. The combined effect of ACh and the phenothiazines (see above; A 3) on rate of contraction.

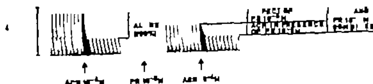


Fig. 1. Reduction in contractile force in an electrically stimulated rat atrium exposed to acetylcholine (ACH) 10^{-4}M before (left) and after (right) treatment with promazine (PR) 10^{-5}M. Promazine 10^{-5}M reduces contractile force to about the same extent as it reduces the response to ACh, and the total reduction is therefore approximately the same in both instances (compare fig. 3)

4 The reduction in work index (= number of beats per minute multiplied by contractile force developed in each beat, Lous 1965) produced by ACh before and after exposure to promazine or thioridazine.
5 The reduction in work index produced by the phenothiazines (see above A 2).
6. The combined effect of ACh and the phenothiazines (see above A 5) on work index.

Drugs:

The drugs used were promazine hydrochloride (Norfarma), thioridazine hydrochloride (Sandoz), acetylcholine chloride and atropine sulphate. Fresh preparations of the phenothiazines were prepared each day while acetylcholine and atropine were prepared from frozen stock solutions.

Results

Contractile force

Acetylcholine gave a dose-dependent reduction in contractile force. The maximal effect (= 100 / of max ACh response) varied from 40 to 100 % of the initial contractile force. The threshold concentration for the effect varied from 5×10^{-8} to 2.5×10^{-7}M, and the maximal effect was obtained with concentrations of either 2.5×10^{-7}M and the maximal effect was obtained with concentrations of either 2.5×10^{-8} or 10^{-6}M When no drug was added before the second dose-response curve in 5 control preparations, two nearly identical curves were obtained for the effect of ACh.

Atropine 10^{-7}M gave a roughly parallel shift to the right of the dose-response curve for ACh in 4 preparations, but did not alter the maximal effect of ACh the antagonism was wholly surmountable. This is the typical alteration in a dose-response curve produced by competitive antagonists.

Promazine 5×10^{-6}M (fig. 2) also altered the dose-response curve to ACh, but in a different way from atropine. The responses to all concentrations of ACh were reduced. The reduction was most pronounced at the highest concentrations of ACh, while the maximal effect of ACh was considerably lower after treatment with promazine 5×10^{-6}M than before. This effect on the dose response curve is generally taken to *indicate unspecific* inhibition.

The reduction in contractile force produced by the phenothiazines made the evaluation of the anticholinergic effects difficult since the initial values changed from the first to the second dose-response curves. The combined effect of ACh and these drugs is determined by the relative magnitudes of the cardio-depressive and anticholinergic actions. Both the negative inotropic and the apparent anticholinergic effects (the shift in the dose-response curve for ACh without taking alterations in initial values into account) depended on the concentration used. Promazine 5×10^{-7}M caused only a negligible alteration in contractile force, but the anticholinergic effect was pronounced.

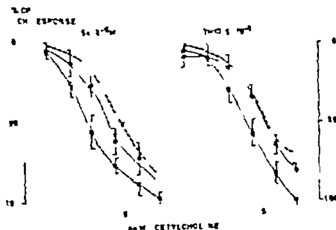


Fig. 2. Reduction in contractile force in isolated electrically stimulated rat atria. — ACh alone (± S.E.M.). Dotted line — ACh in the presence of promazine 5×10^{-6}M (4 atria) or thioridazine 5×10^{-6}M (5 atria). — ACh and phenothiazine combined (± S.E.M.).

The combined effect of ACh and this concentration of promazine was therefore a smaller reduction of contractile force than that produced by ACh alone.

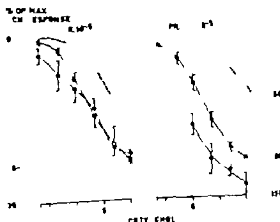


Fig. 3. Reduction in contractile force in isolated electrically stimulated rat atria treated with promazine 10^{-6}M (4 atria) or promazine 2.5×10^{-6}M (10 atria). — ACh alone (± S.E.M.). Dotted line ACh in presence of promazine — ACh and promazine combined (± S.E.M.).

Thioridazine $5 \times 10^{-6}M$ (fig. 2) *and the higher concentrations of promazine* (fig. 3) had greater negative inotropic effects which appeared to balance ($10^{-6}M$ promazine) or even exceed (2.5×10^{-6} promazine) their anticholinergic effects. The cardio-depressive effect of $2.5 \times 10^{-6}M$ promazine was so great that the preparations failed to follow stimulation when more than two doses of acetylcholine had been tested. The control curve is therefore obtained from 10 atria, while each point on the test curves represents 5 atria.

Rate of contraction.

Acetylcholine reduced the rate of concentration in the spontaneously beating atria. The threshold concentration for this effect was higher ($2.5 \times 10^{-7} - 10^{-6}M$) than that required to produce a reduction in contractile force, and $10^{-4}M$ ACh almost invariably produced atrial arrest. In 5 control atria two practically identical consecutive dose-response curves were obtained.

Promazine and *thioridazine* reduced the effect of acetylcholine, but even the lowest concentration used ($5 \times 10^{-6}M$) had such a marked negative chronotropic effect that it balanced the anticholinergic effect (fig. 4).

Higher concentrations of the phenothiazines had even greater negative chronotropic effects and caused atrial arrest even when combined with the lowest ($5 \times 10^{-8} - 10^{-6}M$) concentrations of ACh.

Work index

Acetylcholine caused a reproducible dose-dependent depression in work index in 5 spontaneously beating control preparations. The threshold for

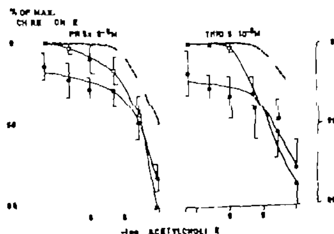


Fig 4. Reduction in frequency in spontaneously beating rat double-atria in the presence of promazine $5 \times 10^{-6}M$ (4 atria) or thioridazine $5 \times 10^{-6}M$ (5 atria). *Symbols* as in figs. 2 and 3.

this effect was the same as the threshold for the negative inotropic effect in the electrically stimulated atria ($5 \times 10^{-9} - 2.5 \times 10^{-7}$M) and the reduction in work index at the lowest concentrations of ACh was entirely due to a reduction in contractile force. At these low concentrations ACh had no effect on rate (see above).

Promazine 5×10^{-6}M and *thioridazine* 5×10^{-6}M had only slight direct effects on the work index (fig. 5). The rate of contraction decreased, but this was compensated for by a corresponding increase in contractile force. Both drugs reduced the effect of ACh – and the combined effect was therefore a smaller depression in work index than that produced by ACh alone.

As mentioned above, higher concentrations of the two phenothiazines caused cardiac arrest when ACh was also given – and the combined effect on work index was therefore greater than that of ACh alone.

Discussion

In the present investigation, promazine and thioridazine altered the dose-response curve to acetylcholine in isolated rat atria in a manner which indicates that the anticholinergic effect of the phenothiazine derivatives is unspecific. The term "atropine-like" should therefore be avoided when referring to the action of the phenothiazines, as it will commonly be taken to indicate specific receptor-blockade.

It should be noted that the anticholinergic activity of these drugs is counteracted by their direct myocardial depressant effects – the combined

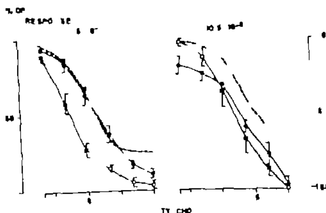


Fig. 5. Reduction in work index in spontaneously beating rat double-atria treated with promazine 5×10^{-6}M (4 atria) or thioridazine 5×10^{-6}M (5 atria). Symbols as in figs. 2, 3 and 4.

effect of acetylcholine and phenothiazines therefore depends on the relative magnitude of these two opposing actions. At low concentrations of the drugs, the anticholinergic property dominates, and the net result is a reduction in the response to ACh. At higher concentrations, however the direct cardio-depressive action is so great as to balance (promazine 10^{-4}M) or even exceed (promazine 2.5×10^{-4}M) the anticholinergic effect. The combined effect is therefore an equal or greater depression of cardiac activity than that caused by ACh alone.

The phenothiazines have been shown to possess membrane-stabilizing properties (LANDMARK 1971b) which may explain their anticholinergic action, since ACh is thought to act mainly through its influence on the ionic permeability of the cell membrane (WILSON & NACHMANSOHN 1954). Due to the membrane stabilizing effect, the drugs will interfere with the movements of ions across the cell membrane It has been stated that this is the way in which another group of membrane-stabilizing agents, the local anaesthetics, exert their anticholinergic effects (FEINSTEIN & PAIMRE 1967).

The negative inotropic effect of ACh is associated with an *increase* in potassium efflux (HOLLAND *et al.* 1952 HARRIS & HUTTER 1956 HOLLAND *et al.* 1959), whereas the negative inotropic effect of the phenothiazines is associated with a *decrease* (LANGSLET 1970 LANDMARK 1971a LANDMARK *et al* 1971) This may partly explain the anticholinergic actions of the phenothiazines, but alterations in the permeability to other ions are probably also involved

ACh reduces the uptake of calcium into the myocardium (GROSSMAN & FURCHGOTT 1964 HODITZ & LÜLLMAN 1964) and the phenothiazines appear to do the same (LANDMARK 1972). This may explain why both ACh and high concentrations of the phenothiazines reduce contractile force, since the availability of intra-cellular calcium appears to be an important factor in the regulation of contractile force.

In the present investigation acetylcholine did not affect the contractile force and atrial rate to the same extent. The negative inotropic effect of ACh had a lower threshold than the chronotropic effect, and whereas ACh nearly always caused atrial arrest, it very rarely produced complete inhibition of the contractile force. As the difference was observed both in electrically stimulated and in spontaneously beating preparations, it cannot be due to the release of ACh from postganglionic cholinergic fibres, which occurs at supra threshold stimulation (VINCENZI & WEST 1965 RUBIN 1970 LANDMARK 1972). It therefore seems possible that the cholinergic receptors in the pace-maker cells differ from those in the remainder of the myocardium, but the differences in the dose response curves may also be due to differences in the ways in which alterations in ionic fluxes influence contractile force and rate.

The effects of ACh and of the phenothiazines on ionic fluxes in cardiac pacemaker cells are not known, because of the technical difficulties involved in studying these cells. As they discharge spontaneously it has been suggested that the ionic movements which regulate the action potential in these cells differ from those in non-pacemaker tissue (SINGER *et al.* 1966). This might explain why the magnitude of the effects of ACh and the phenothiazines on contractile force and rate were different.

The findings presented above may have clinical implications. ACh can produce cardiac arrhythmias *in vitro* (BURN *et al* 1955 HOLLAND & BRIGGS 1959 CONN & LUCHI 1964) and vagal stimulation can do the same *in vivo* (ALEXANDER & PINO 1966). A correlation between anti-arrhythmic and anticholinergic properties certainly seems to exist (SHARMA 1962), but this may be due to the fact that both the occurrance of arrhythmias and the effects of ACh are associated with alteration in membrane permeability The anti-arrhythmic property of the phenothiazines is well documented (COURVOISIER *et al* 1953 MELVILLE 1958 SHARMA & ARORA 1961 MADAN & PENDSE 1963 SINGH & SHARMA 1969 LANDMARK 1971b) but although this may be partly due to their anticholinergic property the unspecific general membrane-stabilizing effect is probably of greater importance. The tachycardia which patients on phenothiazines frequently experience may also be partly due to the anti-cholinergic effects, although the fall in blood pressure which these drugs produce (FOSTER *et al* 1954 SHACKMAN *et al.* 1954) may be more important.

In conclusion it may be said that the present investigation has given some new information about the way in which promazine and thioridazine may influence the cholinergic control of cardiac performance.

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From Sentrallaboratoriet, Dikemark Hospital, Asker Norway
and Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt,
Rikshospitalet, Oslo, Norway

Urinary Metabolites from Rats Given L-DOPA[1]

By

O Borud, T Midtvedt and L. R. Gjessing[2]

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Abstract. Urine from germ-free and normal rats were investigated both before and after oral L DOPA administration. Two neutral L DOPA metabolites were tentatively identified as (N-acetyl)-3-methoxy-4-hydroxyphenylethylamine and 3-methoxy-4-hydroxyphenylethanol. Conjugated dopamine and two other unknown metabolites derived from L-DOPA were detected in addition to 3-methoxy-4-hydroxyphenylacetic acid, 3 4-dihydroxyphenylacetic acid, and 3-methoxy-4-hydroxyphenylethylamine. No significant difference in the excretion of urinary DOPA metabolites was observed between germ-free and normal rats.

Key-words: L DOPA – rat – [(N-acetyl)-3-methoxy-4-hydroxyphenylethylamine] – [3-methoxy-4-hydroxyphenylethanol]

Dopamine was found as a urinary metabolite of D- and L-DOPA in rats by Holtz & Credner (1944) and de Eds *et al.* (1955) showed that oral DOPA given to rabbits resulted in the urinary excretion of 3 4-dihydroxyphenylacetic acid (DHPAA) and homovanillic acid (HVA). Pellerin & d Iorio (1955) and Shaw *et al.* (1957) found dopamine, 3 4-dihydroxyphenylpyruvic acid, HVA and DHPAA as the main urinary metabolites of L-DOPA in rats.

Goldstein & Musacchio (1963) detected N-acetylated 3-methoxy-4-hydroxyphenylethylamine (N Ac MDA) and N-acetylated 3-methoxy-4-hydroxyphenylethanolamine (N Ac NMN) in rat urine, and Tyce (1970) found N Ac-MDA and N-acetylated dopamine as the L-DOPA metabolites of perfused rat liver

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2 Request for reprints. L. R. Gjessing, Sentrallaboratoriet, Dikemark sykehus, 1385 Solberg p. å. Norway

DE EDS *et al* (1955) and SANDLER *et al* (1969) suggested biotransformation of L DOPA to *meta*-hydroxyphenyl compounds by intestinal microorganisms in the rabbit and man respectively and BAKKE (1971a & b) showed that the intestinal flora from rats metabolised L DOPA to *meta*-hydroxyphenylacetic acid and other phenols

We were interested in the effect of intestinal microorganisms on the L DOPA metabolism in rats (BORUD *et al* 1971). In this paper we describe *nine* metabolites in the urine of gnotobiotic (germ-free) and normal rats after oral loads of L DOPA

Methods

The rats were raised and reared as described by MIDTVEDT & TRIPPESTAD (1970). When studying the excretion of phenolic compounds, all the rats were bottle fed for 3 days on a milk diet consisting of commercial dry milk powder (Viking, Nestle, Norway) dissolved in water 1 10 and autoclaved at 120 for 20 minutes. The animals were placed in metabolic cages so arranged as t allow no milk diet to contaminate the urine specimens obtained by means of separators which disposed of the faeces. The diet was withdrawn 12 hours before drug administration. L-DOPA was administered to each rat as single load of 17 mg, i.e. approximat ly 10 mg per 100 gram body weight. The drug was added to 10 ml milk diet and the pH was adjusted to 6,5 with HCl followed by autoclaving. Amino acid analysis of this drug mixture showed no observable decomposition of the L DOPA. The rats completely consumed this 10 ml of milk diet containing the L DOPA within 2 hours, after which they were allowed free access to the milk diet.

Urine samples were collected at room temperature in sterilized bottles containing 5 ml of N-HCl. The bottles were replaced every 12 or 24 hours and stored at −20 until the determination of L DOPA compounds.

Creatinine in urine was estimated according to Jaffe's method.

Hydrolysis / urine was done at 37° for 24 hours with β-glucuronidase-arylsulphatase made from Helix pomatia (Calbiochem. U.S.A.) at pH 5 5 after precipitating the sulphate with $BaCl_2$ (BORUD & GJESSING 1970) Acid hydrolysis was done at pH 1 with HCl, 100 20 min.

Phenolic acids were detected according to ARMSTRONG *et al.* (1956).

Phenolic amines were determined by the method of KAKIMOTO & ARMSTRONG (1962) or by high voltage electrophoresis and chromatography according to GJESSING (1963).

Neutral phenolic compounds were extracted from urine with ethylacetate at pH 7.6 and detected on bidimensional chromatograms according to BORUD & GJESSING (1970) where thin-layer chromatography on silicagel with thylacetate is also described. Thin-layer cellulose plates were made according to TAKAHASHI & GJESSING (1971).

Keto acids were precipitated with 2,4-dinitrophenylhydrazine according to SMITH (1960).

Staining reagents: Diazotised *p*-nitroaniline and diazotised sulphanilic acid were made according to ARMSTRONG *et al.* (1956), Gibb's reagent as described by SMITH (1960). Ninhydrinspray was made by dissolving 0 2 g in 100 ml acetone (stock solution kept at 6) and 5 ml 2,4,6-collidine was added immediately before use. Ethylenediamine-ferricyanide was made according to TAKAHASHI & GJESSING (1971).

Acetylation of amines was done according to the method of GOLDSTEIN *et al.* (1961). *Standards and abbreviations.* 3-methoxy-4-hydroxyphenylacetic acid, (HVA), 3-methoxy-4-hydroxyphenylethylamine, (MDA) 3-methoxy-4-hydroxyphenylethanol, (VE), L-3 4-dihydroxyphenylalanine, (L-DOPA), 3,4-dihydroxyphenylethylamine, (dopamine), 3,4-dihydroxyphenylacetic acid, (DHPAA), were all commercial products. (N-acetyl)-3-methoxy-4-hydroxyphenylethylamine (N Ac MDA) was synthesized.

Results

Three germ-free, five monocontaminated and four normal rats were studied on a controled milk diet and after oral loads of 17 mg of L-DOPA. All the rats excreted the same metabolites in similar amounts in the urine. On the diet the rats excreted mainly para-tyrosine metabolites (BORUD *et al* 1971) After L-DOPA they excreted large amounts of HVA, DHPAA, MDA, dopamine and the unknown compounds A, B C, shown on the chromatogram in fig. 1 The addition of 2,4-dinitrophenylhydrazine to fresh rat urines did not change this pattern, and the keto acids 3-methoxy-4-

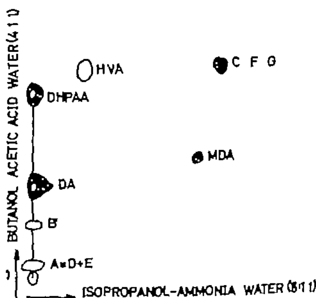


Fig. 1. *Two dimensional urinary chromatogram made from rat loaded with L-DOPA (urine corresponding to 25 μg of creatinine).*

Open circles: Diazotised paranitroaniline positive spots before hydrolysis: homovanillic acid (HVA) 3,4-dihydroxyphenylacetic acid (DHPAA) dopamine (DA) and two unknown spots A and B. *Hatched area:* Spots visible after enzymatic hydrolysis: increased DHPAA and dopamine, disappearance of A and B appearance of 3-O-methyldopamine (MDA) and spot C, see table 1 and 2.

hydroxyphenylpyruvic acid and 3 4-dihydroxyphenylpyruvic acid were not detected in rat urines after L DOPA as the 2,4-dinitrophenylhydrazones. These acids were only minor L-DOPA metabolites in the urine (less than 5 / recovery from L DOPA ingested) The excretion of phenolic compounds was almost normalised twelve hours after DOPA treatment.

Following DOPA treatment the rats excreted 100–300 µg HVA per mg of creatinine or 0.5–2 mg per 24 hours (3–12 / recovery from L-DOPA) and only a small amount of the acid was conjugated.

The urinary DHPAA was excreted at the same level as HVA, but this acid was partly conjugated, see fig. 1

The red spots A and B were major metabolites in the urine of germ-free as well as of conventional rats after DOPA administration. These spots disappeared when the urine was hydrolysed enzymatically or with hydrochloric acid, but both dopamine and MDA increased and spot C appeared, as shown in fig. 1

Spot A was purified from the urine by high voltage electrophoresis and chromatography in a formic acid run. Most of the red spot A, designated as compound D migrated into the electrophoresis like tyrosine and was different from dopamine. Another small amount of A, designated as compound E, did not migrate at all.

Substance D was isolated from the paper concentrated and enzymatically hydrolysed in 1 N-NaAc at pH 5 After hydrolysis the extract contained a compound similar to dopamine. This dopamine-like compound had the same high voltage electrophoretic value and the same chromatographic behaviour in 4 different runs as standard dopamine, see table 1 The colour reactions with 4 different spray reagents were also identical with dopamine. Table 1 also shows the Rf values and the colour reactions of *meta*-hydroxyphenyl acetic acid in order to demonstrate the dissimilarities of spots A and B with this acid.

The amount of free dopamine in 5 rat urines in the first twelve hours after L-DOPA administration was 4–32 µg per mg of creatinine or a total of 0.02–0 19 mg (mean 0.11 mg) The total dopamine excretion measured after enzymatic hydrolysis was 32–427 µg per mg of creatinine or a total of 0 19–2.56 mg (mean 1 03 mg) in twelve hours. The total recovery of dopamine from L-DOPA in the five rats was 1.5–19.8 / (mean 8.7 /)

Spot C in fig. 1 was isolated and purified by enzymatic hydrolysis of the urine containing 5 mg of creatinine, extracted at neutral pH and examined by paper chromatography in acidic and alkaline runs. The chromatographic properties and colour characteristics of this spot are shown in table 2. Thin-layer chromatography on silicagel showed that spot C was split into two compounds, F being the major and G the minor part. Thin-layer chromatograms showed that F had the same R_f value as standard N-Ac MDA,

Table 1

R_f and E_f values and colour characteristics of different compounds from rat urine after L DOPA loading.

Compound	R_f values: But. Ac.	R_f values: Isopr form.	R_f values: Isopr NH_3	R_f values: Thin-layer	E_f values Electrophoresis cm/90 min.	Colour reactions: DzPNA	Colour reactions: DzSA	Colour reactions: Nin	Colour reactions: Gibb's	Colour reactions: F reagent Fluorescence
Tyrosine					16	blue-purple	purple	bluegrey		
		D E			D E					
A (=D+E)	0,05	0,09+0 40	0	0	16 (+0)	red	yellow	bluegrey	blue	+
D	0,05	0,09	0	0	16	red	yellow	bluegrey	blue	+
E	0,05	0,40	0	0	0	red	yellow	+	blue	+
D hydrolysed	0,36	0 46	decomp.	0,57	19,5	purple		bluegrey	grey	green
D hydrolysed + dopamine	0,36	0,46	decomp.	0,57	19,5	purple		bluegrey	grey	green
dopamine	0,36	0,46	decomp.	0,57	19,5	purple		bluegrey	grey	green
B	0,22	0,40	0	0	0	red	yellow	+	blue	+
m-hydroxy phenylacetic acid	0,91		0,37		0	red	yellow	+	blue	+
p-hydroxy-phenylacetic acid	0,91		0,35		0	red	yellow	+	blue	+

Paper chromatographical runs: But. Ac.. n-butanol-acetic acid-water (4-1 1), Isopr form. isopropanol-formic acid-water (8-1 1), Isopr NH_3: isopropanol-ammonia-water (8-1 1), Thin-layer on cellulose with n-butanol-acetic acid-water (12 3-5), Electrophoresis: buffer acetic acid-formic acid-water pH=2 with 2500 volt and 1½ hour

Spray reagents: DzPNA: diazotised p-nitroaniline, DzSA. diazotised sulphanilic acid, Nin. ninhydrin –2,4,6-collidine, Gibb's: 2,6-dichloro-benzoquinone-4-N-chloroimine, F reagent: ethylenediamine-$KFe(CN)_4$ and ultraviolet light.

Hydrolysis was done enzymatically with β-glucuronidase + arylsulphatase in 1 N-NaAc at pH=5 37 for 4 hours.

Abbreviations see fig. 1

Table 2

R_f and E_f values of neutral compounds from hydrolysed rat urines after L-DOPA administration.

Compound	R_f values				E_f values Electrophoresis cm/90 min.	Colour reactions		
	But. Ac.	Isopr NH_3	Thin-layer			DzPNA	Nin	Gibb's
			G	F				
C (=G+F)	0,89	0,86	0,52	0,12	0	bluegrey	bluegrey	grey
G	0,89	0,86	0,52		0	bluegrey	+	grey
VE	0,88	0,84	0,52		0	bluegrey	+	grey
F	0,89	0,86	0,12		0	bluegrey	bluegrey	grey
F hydrolysed	0,61	0,75	0,05		20	bluegrey	bluegrey	grey
F hydrolysed + MDA	0,61	0,75	0,05		20	bluegrey	bluegrey	grey
MDA	0,61	0,75	0,05		20	bluegrey	bluegrey	grey
N-Ac MDA	0,89	0,85	0,12		0	bluegrey	bluegrey	grey
N Ac-MDA hydrolysed	0,61	0,75	0,05		20	bluegrey	bluegrey	grey

Chromatographical runs: Thin-layer – on silicagel with ethylacetate, the other runs, electrophoresis and spray reagents were as described under table 1. Abbreviations see fig. 1 and under methods.

and G the same R_f value as standard 3-methoxy-4-hydroxyphenylethanol (VE) see table 2.

Substance F eluted from thin-layer plates, had the same chromatographic properties in 3 solvent systems and the same electrophoretic migration as standard N Ac-MDA, as shown in table 2. The colour reactions with ninhydrin, diazotised *p*-nitroaniline and Gibb's reagent were also similar to the standard N Ac-MDA.

Synthetic N Ac MDA was split into MDA by *alkaline hydrolysis* in 2 N-NaOH at 100° for 20 minutes, as shown in table 2. Standard VE was partly decomposed under these conditions. N Ac MDA was not split into free MDA by acidic hydrolysis at pH 1

Compound F was *hydrolysed in alkali* and the products were run on paper chromatograms with and without the addition of standard MDA. F disappeared during hydrolysis and a compound appeared with the same chromatographic properties and the same colour reactions as MDA, see table 2. These findings indicated that compound C in fig. 1 consisted mainly of N Ac MDA and contained only a small amount of VE.

Spot B in fig. 1 had the same colour reactions as spot A with Gibb's reagent, diazotised *p*-nitroaniline and sulphanilic acid, but the ninhydrin reaction was negative. Urine containing compound B was hydrolysed enzymatically or with hydrochloric acid after which spot B disappeared. Compounds B and E, which is the minor part of A, were purified by high voltage electrophoresis and chromatography B and E did not migrate on electrophoresis, and they had the same R_f values in the alkaline run and also in isopropanol-formic acid-water Purified B and E was enzymatically hydrolysed, and the compounds disappeared from the solution. We were not able to detect DOPA, dopamine or DHPAA as hydrolytic products from B and E.

The rats excreted less than 0.2 / of free + conjugated L DOPA or 3-0-methyl-DOPA in the urine, whereas we found a 0 1–1 / recovery of L DOPA and 3-0-methyl-DOPA in normal man and in patients with Parkinson's disease (O Gorman *et al* 1970 and unpublished results)

Discussion

Pellerin & D'Iorio (1955) injected D,L DOPA 2-C^{14} into rat and recovered in the urine up to 26 / as dopamine, 19 / as DOPA, 15 / as 3 4-dihydroxyphenylpyruvic acid, and 7 / as dihydroxyphenylacetic acid. They found dopamine as a major metabolite from the injected DOPA, as we found after oral administration.

Compound D in the red spot A was tentatively identified as dopamine

conjugated in the *para*-hydroxy position. We assume that the dopamine was conjugated with sulphate because compound D was split both by acid and by enzymatic hydrolysis. This dopamine-conjugate was not found in human urine after L DOPA treatment (O GORMAN *et al.* 1970).

50 / – 75 / of the urinary dopamine in man after L-DOPA was found to be conjugated (unpublished results) whereas rats excreted up to 90 / of the dopamine in a conjugated form. These results indicate that the rat ex cretes a higher percentage of dopamine in a conjugated form than man. Another interesting difference between man and rat was that the rat excretes dopamine as one of the major metabolites from L DOPA, while man only excretes dopamine as a minor compound. The conjugation system is perhaps more effective in rats thus facilitating dopamine excretion.

The 3-methoxy-derivative of dopamine, MDA, was also found mainly in a conjugated form. This amine was probably conjugated in a similar way as dopamine since acidic and enzymatic hydrolysis of rat urines resulted in the appearance of MDA.

Another conjugation mechanism for dopamine and MDA in rats (GOLDSTEIN & MUSACCHIO 1963) and in man (SEKERIS & HERRLICH 1963) is acetylation of the amino group of dopamine or MDA. We found compound F in rat urine, tentatively identified as N Ac-MDA. This compound was the major part of total urinary MDA-conjugates as shown in fig. 1 MDA was present in smaller amounts than the blue-grey spot C. Both MDA and N Ac MDA were most likely conjugated with sulphate as they were detectable in the urine by enzymatic as well as by acidic hydrolysis. Our finding of N-Ac MDA in the urine of germ-free rats supports this finding (TYCE 1970) She emphasized that N-acetylation is important in the metabolism of DOPA in the rat.

An N-acetylated phenolamine and the corresponding phenol alcohol are difficult to separate by paper chromatography or electrophoresis (GOLDSTEIN & MUSACCHIO 1963 BORUD & GJESSING 1970). In the urine of patients suffering from neuroblastoma or from patients given L-DOPA, one has to be careful to distinguish between these four neutral metabolites, 3 4-dihydroxyphenylethanol, N Ac DA, VE, and N-Ac MDA. CALNE *et al* (1969) detected small amounts of VE in the urines of patients with Parkinson's disease on L DOPA treatment, and we also found small amounts of this metabolite in the urine of normal subjects on L DOPA (O GORMAN *et al* 1970). CALNE *et al.*, however did not mention N-Ac-MDA. Our results indicate that human subjects excrete some VE but very little N Ac MDA after L DOPA administration, while the rat excretes significant amounts of N Ac MDA, but very little VE. These results indicate that the metabolism of L DOPA is different in rats and man and it would be interesting to know whether this difference has any physiological significance.

We were not able to identify one of the major DOPA metabolites, i. e. spot B in fig. 1, but this spot may be a conjugated dihydroxy compound (BAKKE 1971b) TYCE (1970) in her perfusion experiments, found a volatile compound and it is possible that this compound is B

B was split by hydrolysis in the same way as the conjugated dopamine, but we were not able to detect any of the usual DOPA metabolites or DOPA itself in the hydrolysate. Spot B was negative to the ninhydrin spray which excludes conjugated N-acetylated dopamine.

CALNE *et al* (1969) suggested that minor pathways of L DOPA metabolism could be important in man, and they detected meta-hydroxyphenylacetic acid as one possibly important metabolite formed by intestinal microorganisms. We did not find this compound on our chromatograms either in germ-free or in normal rats on a milk diet.

In a conclusion there was no difference between germ-free and normal rats with regard to their urinary pattern of DOPA metabolites when given a controlled milk diet.

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From the Department of Pharmacology
University of Copenhagen, 20 Juliane Maries Vej DK-2100 Copenhagen Ø,
Denmark

Age Dependence of the Effect of Oestrogenic Treatment on the Acid Mucopolysaccharide Pattern in the Skin of Mice

By

Nina Grosman

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Oestrogenic treatment of mice causes a significantly increased content of water and acid mucopolysaccharides (AMPS) (e.g. hyaluronic acid (HA)) in the subcutaneous connective tissue ground substance (HVIDBERG *et al.* 1963 LANGGÅRD & HVIDBERG 1969 GROSMAN *et al* 1971). Treatment of mice of a similar age provokes reproducible changes of the ground substance, and consequently this tissue serves as a useful ground substance "model" (HVIDBERG *et al* 1963) Previously published results by other investigators indicate that the response to oestrogenic treatment is unaffected by the age of the animals (WARREN & FAGAN 1960 SOBEL & COHEN 1970) This is not in agreement with our own experience and the purpose of the present study was to quantitate the effect of the treatment on two different age groups of mice.

White male mice (NMRI/BOM strain) weighing 33 4 ± 2.7 g (mean ± S.D) 7–8 weeks old and thus sexually mature (FRIIS 1972, personal communication) were used. The effect of oestrogenic treatment on this group of mice was compared with the results of a previously published investigation on younger mice (weighing 27 6 ± 1 4 g, 5–6 weeks old and not sexually mature (FRIIS 1972, personal communication)), of the same strain (GROSMAN *et al.* 1971) The mice were treated and the AMPS isolated and fractionated by hydrolysis with papain, cetylpyridinium chloride (CPC) precipitation and cellulose column fractionation as previously described (GROSMAN *et al* 1971).

Table 1 *shows that the content of water* and AMPS is almost identical in the skin of the two control groups. In contrast to this finding the response to oestradiol treatment is extremely different in the two groups of mice. Both

Table I

The effect of oestradiol treatment on the tissue content of water and AMPS.
The values are given as the mean ± S.E.M. per gram dry fat free tissue.

	Number of mice	Age of mice weeks	Water content g	Total CP AMPS	1 % CPC eluate	HA	Chondroitin sulphates
Control-mice	14	5–6	[illegible].70 ± 0.03	6.67 ± 0.24	1.26 ± 0.14	2.70 ± 0.14	3.71 ± 0.10
Control-mice	8	7–8	2.58 ± 0.08	6.08 ± 0.28	1.53 ± 0.08	2.84 ± 0.16	3.17 ± 0.11
P (C–C)			> 0.1	> 0.1	> 0.1	> 0.1	< 0.005
Ovex-mice	14	5–6	5.15 ± 0.30	28.1 ± 3.0	10.8 ± 1.7	14.6 ± 1.2	4.20 ± 0.27
Ovex-mice	11	7–8	3.88 ± 0.16	17.8 ± 1.4	7.03 ± 1.3	9.68 ± 0.52	3.45 ± 0.31
P (O–O)			< 0.005	< 0.005	> 0.1	< 0.001	> 0.05
P (O–C)			< 0.001	< 0.001	< 0.001	< 0.001	> 0.1

µmol uronic acid.

By means of Student's t-test the difference was tested between the two control groups (P (C–C)), the two ovex groups (P (O–O)) and between ovex and control mice 7–8 weeks old (P (O–C)).

The results from the 5–6 weeks old mice have previously been published (GROSSMAN *et al.* 1971).

From the Department of Pharmacology
University of Copenhagen, 20 Juliane Mariesvej, DK 2100 Copenhagen Ø Denmark
(Head. Professor J Schou, M.D.)

A Simple Method for the Fluorimetric Determination of 5-Hydroxyindoleacetic Acid in Human Urine

By

Inge K. Genefke

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Key-words. 5-Hydroxyindoleacetic acid, fluorimetric determination – human urine.

5-hydroxyindoleacetic acid (5-HIAA) is the principal metabolite of 5-hydroxytryptamine (5-HT) in human urine. Several methods for the determination of 5-HIAA in urine have been reported (UDENFRIEND *et al.* 1955a, MUSTALA *et al* 1964 HANSON & SERIN 1955 CONTRACTOR 1966 KORF & VALKENBURGH-SIKKEMA 1969) Until now colorimetric technics have usually been used. Most procedures are based on the reaction between 5-hydroxyindole compounds and 1-nitroso-2-naphthol which forms a violet chromophore in nitrous acid (UDENFRIEND *et al.* 1955b).

The present method is based on the native fluorescence of 5-HIAA in 3 N HCl. Interfering substances are eliminated with Florisil (DRIUX 1968). Blanks are obtained by oxidation with periodate (KORF & VALKENBURGH-SIKKEMA 1969).

Urine is adjusted to about pH 1 by the dropwise addition of concentrated HCl. Four ml of urine is shaken for 5 min. with 1 g Florisil [Florisil 60–100 mesh (magnesia silicat) (Fluka) 0 15 M phosphate buffer pH 7 1/15 M KH_2PO_4 and 1/15 M-Na_2HPO_4 (39 ml plus 61 ml). Ethyl Acetate, analytical grade. Hydrochloric acid, analytical grade. 5-HIAA standards (Sigma) 10 mg 5-HIAA dissolved in 100 ml HCl 0 01 N Sodium Periodate solution 0 02 / (The British Drug House Ltd.) NaCl, analytical grade. Redistilled water is used in all solutions.] and then centrifuged for 5 min. at 1000 x g. Two ml of the supernatant is added to 1 g NaCl and shaken for 5 min. with 5 ml of ethyl acetate. Four ml of the organic phase is shaken for 5 min. with 1.5 ml of phosphate buffer The organic phase is discarded and 1 ml of the aqueous phase is adjusted to 3 N with concentrated HCl Internal standards, prepared

by adding 2.5 5 0 or 10 0 µg of 5-HIAA to one ml urine, are carried out through the entire procedure. The fluorescence of the sample and the internal standards are measured at 545 nm (uncorrected) in an Aminco-Bowman spectrofluorometer activating at 310 nm (uncorrected). The actual concentration of 5-HIAA in the sample is calculated from the fluorescence of the sample after deduction of the blank by dividing with the fluorescence produced by 1 µg 5-HIAA measured on the internal standard curve for the sample (comp. fig. 1). Blanks are obtained by adding 100 µl of 0 02 / sodium periodate to each sample after the fluorescence has been measured. The concentration of 5-HIAA in different samples of urines cannot be calculated from the same standard curve, as the slope of the internal standard curve differs between samples of urine.

All samples are run in duplicate.

Activation and fluorescence spectra of authentic 5-HIAA (2.5 µg/ml), a urinary extract, a urinary extract with an internal standard (2.5 µg 5-HIAA/ml urine) and a blank obtained by periodate oxidation are shown in fig. 2, A and B The spectra of authentic 5 HIAA and urine extracts are essentially the same.

The internal standard curve for 5-HIAA in normal human urine is linear within the concentration range investigated (2.5 10 µg 5-HIAA/ml of urine, fig. 1). The calculation of the 5-HIAA content in the samples is described above.

The reproducibility of the method was tested by double determinations (table 1)

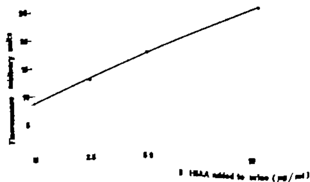


Fig. 1. The fluorescence (*Ordinate:* arbitrary units) of urinary sample (O) samples from the same urine t which have been added 2.5 5.0, and 10 µg 5-H *Abscissa.* 5-HIAA added to urine (µg/ml).

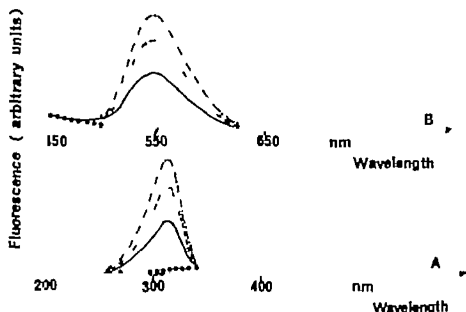


Fig. 2. Activation spectrum (A) (fluorescence measured at 545 nm) and fluorescence spectrum (B) (activation t 310 nm) of authentic 5-HIAA – — — — as urinary extract ———— a urinary extract with an internal standard (2.5 µg 5-HIAA/ml urine) — — – – – — and an urinary extract after destroying 5-HIAA

As shown in table 2 a good correlation exists between the present fluorimetric method when compared to the colorimetric method of UDENFRIEND *et al.* (1955) as modified by DREUX & DELANEUX (1964). The mean values from 6 samples of normal urine averaged 4 6 and 5 0 µg/ml respectively

In order to see whether there is also a linear relationship in urine con-

Table 1

Sample No.	5-HIAA (µg/ml)	Duplicate samples
1	4 7	5.0
2	6.4	6.4
3	5.2	5.2
4	2.3	2.2
5	4 1	4.1

Double determinations of 5-HIAA µg/ml urine determined by the fluorimetric method.

Table 2

Colorimetric method	Fluorimetric method
4.6	4 1
3.3	3.4
6 4	5.6
5 2	4.9
5.8	5.5
4.8	4.1

5-HIAA (mg/ml urine) determined by a colorimetric method compared with the lues obtained by the fluorimetric method.

taining less 5-HIAA than in normal urine, 5-HIAA was removed from the urine The urine was saturated with NaC1 and washed with a double volume of ethyl acetate When 5-HIAA was added to the samples a linear relationship between concentration and fluorescence was obtained (fig. 3)

The method presented for the determination of 5-HIAA in urine was found to be satisfactory as to specificity sensitivity and reproducibility The crite rion used for specificity was identical spectra for apparent 5-HIAA extracted from urine and the authentic compound. The fluorescence in 3 N-HCl at 545 nm (uncorrected), activating at 310 nm (uncorrected) is specific for the 5-hydroxy- and 5-methoxy-indoles (QUAY 1963).

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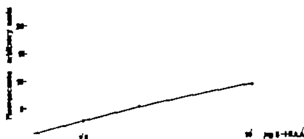


Fig. 3 The fluorescence (*Ordinate* arbitrary units) of 2.5, 5.0 and 10 0 μg 5-HIAA added t one ml urine from which 5-HIAA has previously been removed by washing with ethyl acetate. The samples have been carried through the entire

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From the Department of Pharmacology and the Section of Toxicology University of Copenhagen, 20 Juliane Maries Vej, DK 2100 Copenhagen Ø Denmark

Tissue Concentrations in a Case of Acute Mercuric Chloride Poisoning

By

Anni Steentoft

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Key-words: Mercury – f tal poisoning – tissue concentrations.

In the past 10 years only one case of acute mercury poisoning has been investigated in our department. Since only few reports are found in the recent literature, we thought it relevant to publish our results, obtained by the method described by SIMONSEN (1953)

The mercury is extracted as the dithizonate following reflux digestion of the biological material (2 g). A spectrophotometric measurement of the dithizone extract is performed before and after iodide treatment, by which the mercury-dithizonate complex is destroyed. The difference between the two readings at 600 mμ is used for the calculation of the mercury content in

Table 1

Concentrations of mercury in different organs of patient, who died from acute mercuric chlorid poisoning

	Conc of mercury μg/g (ml)	Total amount of mercury mg
Gastric contents	14386	31.7
Contents of small intestine	482	72.3
Contents of large intestine	317	3.8
Brain	3	3.7
Blood	22	(110 mg/5 l)
Liver	56	77.3
Spleen	56	4.2
Pancreas	188	24.4
Muscl	6	(150 mg/25 kg)
Kidney	136	44.2

the sample by comparison with a standard curve obtained by measuring solutions with known amounts of mercury

The recovery of known amounts of mercury 5 μg and 25 μg (as a mercuric salt) added to homogenized liver (2 g) not containing mercury is measured to 80 / and 84 / respectively

In our case a man aged 53 years died a few hours after taking a white powder identified as mercuric chloride. The concentrations of mercury in the different organs are shown in table 1 The results are the mean values from two dithizone extractions

The results of the chemical analyses are in agreement with mercury poisoning as the cause of death. The high concentration in the pancreas is remarkable. In comparison with this case KLENDSHOJ & REJENT (1966) have published a case of mercury poisoning with 4.5 g of bichloride of mercury where the patient died on the 8th day after admission. The method used is the same as in the present case. They found 0.8 μg/ml blood 1.1 μg/g brain, 33.2 μg/g liver and 46 7 μg/g kidney

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